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16 OCT 1997

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Patents ADP number *(if you know it)* 7739450001

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PARTICLES

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PARTICLES

The present invention relates to the targeting of biological cells e.g. for the purpose of analysing for the presence and/or amount thereof in a particular sample or for the purposes of drug delivery.

Biological cells is a term widely used to describe living cells as the entities which substantially comprise the body of a wide range of living organisms, such as micro-organisms (e.g. bacteria, protozoa, fungi, algae), plants and animals. Biological cells may be provided as single cells, as individual cells in a suspension or as cells that may be associated in the form of multicellular organisms or the tissues and organs therein.

It is desirable to be able to monitor for, and/or identify, specific types of cells in a sample. Such cells may be within inanimate material (for instance pathogenic organisms contaminating a food stuff or water supply) or may be mixed with other types of cells (for instance a microbiological infection of a multicellular organism or a cancer cell within a patient).

The metabolic activity of a cell causes changes in the extracellular environment and conventionally such changes have been directly measured by placing a measuring device in, or close to, a medium containing the cells of interest. Thus, for examples, the metabolic activity of cells has been measured by monitoring change in pH, typically resulting from the release of carboxylic acids (such as lactic, acetic acids), changes in gases (such as carbon dioxide and ammonia and their dissolution to form ionic electrolytes) or other protonatable groups (such as amines and amino acids). Typically pH is measured by immersing a pH electrode in the medium continuous with the biological cells. Similar physiological activities have been measured by immersing a conductivity electrode in the said medium, in so far as such measurement results from the changes in the concentration and charge of ionic electrolytes in the said medium.

Alternatively, the metabolic activity of cells has been measured with a redox electrode, such as a platinum electrode, which measures the ability of the medium continuous with the cells to donate electrons to, or accept electrons from, an electronic circuit of which the electrode forms one part. Thus, the cells in contact with a medium are the variable part of an electrochemical cell also contacted by at least 2 electrodes, which may measure pH, conductivity or redox potential (in a potentiometric circuit) or Faradaic current (in an amperometric circuit).

The metabolic activity of a cell may also be measured indirectly. For example, a dye may be introduced into the medium containing cells such that the optical properties of the dye (e.g. absorption or fluorescence at a particular wavelength of light) change in proportion to changes in the medium (such as pH or redox potential). Such optical and some electrochemical measurements are also coupled to the activities of enzymes produced by cells. For instance, the activity of hydrolytic enzymes, such as proteases, may be measured by their action on chromogenic or fluorogenic substrates.

There are various inadequacies associated with these conventional measurement techniques. For instance, the magnitude of change in the medium (i.e. the metabolic activity being monitored) wrought by low numbers of cells (typically where cell concentration is less than 10^4 to 10^5 cells per ml) is often insufficient to produce a measurable effect. Furthermore similar changes in the medium may be mediated by other cells (which are not of interest) or by materials also present in the medium resulting in multiple or interfering contributions to the measured change, and poor specificity towards the desired measurement. These drawbacks of sensitivity and specificity are especially commonplace in non-specialised devices and assays which are used outside of a specialist laboratory to perform routine measurements.

The detection of micro-organisms such as bacteria in food and/or environmental samples typically requires the presence of at least 10^4 organisms for measurement over a period of 1 to 4 hours. Theoretically at least, 1 organism or cell can be measured if that

cell grows and divides exponentially to a minimum threshold concentration of organisms required by the detection means, however this may take many hours.

Apart from the purposes of analysis, there are other procedures where recognition of cells (e.g. by targeting) are important. One such example is the case of drug delivery. Targeted delivery of a drug to a specific cell type is highly desirable as it removes the need for systemic administration and thereby reduces the risk of non-specific effects or undesirable side effects in non target cells.

Specific agents may be targeted to a cell for the purposes of drug delivery or analysis in a number of ways. For instance, antibodies recognising cell specific antigens may be used to a target a cell. Such antibodies may be directly or indirectly linked to other agents such as detectable labels or a therapeutic agent. Recently particles such as liposomes have been provided with antibodies to form immunoliposomes. These immunoliposomes are useful for targeting any substance contained within the liposome to a cell.

Once a species has been targeted to a cell there is usually a need to induce it to the cell to undergo a reaction, or be released, in order that it may exert its desired effect. For instance, when a cell is targeted for the purposes of monitoring for the presence of that cell in a sample, the species may be a detectable label which needs to be released and/or activated in the vicinity of the cell. In the case of a drug delivery system, the drug may need to be released and/or activated at the target site. One problem associated with known targeting particles, for instance immunoliposomes, is that they have limited utility because release or activation of their contents often depends upon cell mediated endocytosis and subsequent rupture of the liposome inside the cell whereas many substances targeted to a cell may need to be released extracellularly (e.g. ligands of cell surface receptors) in order that they may exert a desired effect. When such events do not occur the release of the substance may not be effective.

It is an object of the present invention, to obviate or mitigate the abovementioned disadvantages.

According to a first aspect of the present invention, there is provided a particle capable of being targeted to a cell type of interest, said particle incorporating a species to be targeted to the cell and being such that the species is activated (as defined herein) in response to a predetermined metabolic signal from the targeted cell.

Activation of the species may take a number of forms such as release from the particle and/or conversion (chemical or otherwise). Conversion may occur within the particle or without.

We have found that particles according to the first aspect of the invention are very useful for targeting a species to a cell and then activating that species in the vicinity of the cell. This reaction may be highly sensitive and/or highly selective depending upon the nature of the metabolic signal from the targeted cell.

It is a common characteristic of biological cells that they contain agents, such as enzymes, that perform reactions and other processes, such as the transport of electrons, protons, ions, chemical and biochemical species. Such reactions and processes are often termed metabolism. Metabolism within a cell often results in a change in the environment surrounding the cell, which environment may also affect the metabolism within the cell. These phenomena will be understood as key aspects of the physiology of the biological cell and are termed herein as metabolic signals.

Suitable particles include any size of particle not too large to mix with and disperse readily in samples containing cells. Particles should be small enough such that they will not sediment in aqueous suspension. Particles may be from a few tens of microns in diameter down to sub-micron sized particles.

The particles may be prepared from polymers and comprise structures which change in response to the metabolic signal. However preferred particles are vesicles consisting of lipid bilayer bound cavities. For instance, the vesicles may be formed from liposomes. The species may be retained within the internal cavity formed by the lipid bilayer.

The particle may be adapted such that the species is activated in a number of ways.

Vesicle particles may be adapted such that the permeability of the lipid bilayer may be regulated. The permeability may be increased in response to a predetermined metabolic signal by incorporating cytolytic agents into the vesicle. For instance, special lipids may be incorporated into the vesicle such that their charge and structure may be altered (e.g. by proton or electron transfer reactions) in response to the metabolic signal. Examples of such lipids include dioleoylphosphatidyl (DOPE) / oleic acid; 1,2 dioleoyl-3-succinyl-glycerol and 1,2 diacyl-3-succinyl-glycerol. This alteration in charge and structure may result in a change in permeability of the lipid bilayer. This permeability change may alter the intravesicular chemical environment and thereby allow the chemical alteration and/or release of substances contained within the vesicle.

A more preferred means of making particles (vesicular or otherwise) responsive to the metabolic signal is to incorporate cytolytic agents into the particle which are responsive to the metabolic signal. Preferably such agents are peptides. In the case of vesicles, these peptides may mediate the opening of pores or channels within the lipid bilayer to allow molecules to enter into the vesicle and thereby cause the chemical alteration of the substance contained therein. Alternatively, or additionally, the opening of the pores or channels within the lipid bilayer may allow the release of the substance into the extravesicular environment. The cytolytic agents, particularly peptides, may be responsive to a chemical or biochemical released from the cell. For instance, the peptide may be an ion channel which "opens" in response to ions (e.g. H^+, Na^+, Cl^-, HCO^-, K^+

etc.). Alternatively the ion channel may respond to the binding of larger molecules derived from the targeted cell (for instance a growth factor, a component of the extracellular matrix of mammalian cells or capsule polysaccharides of micro-organisms). It is also possible to genetically engineer a peptide cytolytic agent such that it will be responsive to any predetermined metabolic signal from a selected cell.

Peptide cytolytic agents of vesicles are preferably integral proteins of the lipid bilayer (i.e. the peptide spans the lipid bilayer). However it will be appreciated that the peptide may interact with the bilayer in other ways (e.g. non covalently attached to the outer lipid layer).

Preferred cytolytic agents for making particles responsive to a metabolic signal are listed in Table 1.

TABLE 1: CYTOLYTIC AGENTS

Aerolysin
Amphotericin B
Aspergillus haemolysin
Alamethicin
A-23187 (Calcium ionophore)
Apolipoproteins
ATP Translocase
Cereolysin
Colicins
Detergents (Triton, Brij, Cetrimides)
Direct lytic factors from animal venoms
Diptheria toxin
Filipin
GALA
Gramicidin
Helical erythrocyte lysing peptide (HELP)
Hemolysins
Ionomycin
KALA
LAGA
Listeriolysin
Melittin
Metridiolysin
Nigericin
Nystatin
P25
Phospholipases
Polyene Antibiotics
Polymixin B
Saponin
Sodium fusidate
Staphytlococcus aureus toxins (α,β,χ,δ)
Streptolysin O
Streptolysin S
Synexin
Surfactin
Tubulin
Valinomycin
Vibriolsin

Preferred particles comprise a liposome with a peptide cytolytic agent in the lipid bilayer. The particle may comprise 1mg of peptide for every 1 - 1,000 mgs of lipid, preferably 1mg of peptide for every 10 - 100 mgs of lipid and most preferably 1mg of peptide for every 30 - 60 mgs of lipid. However it will be appreciated that the exact ratio of peptide to lipid will depend upon the specific characteristics required in the liposome particle being formed.

A preferred peptide cytolytic agent is N, Myristic-GALA.

N, Myristic-GALA has an amino acid sequence of W-E-A-A-L-A-E-A-L-A-E-A-L-A-E-H-L-A-E-A-L-A-E-A-L-E-A-L-A-A (where W is Tryptophan, E is Glutamic Acid, A is Alanine, L is Leucine and H is Histidine). The Myristic acid is reacted with the N-terminal (Tryptopan; W) to give N-Myristic GALA.

It will be appreciated that other cytolytic peptide may be used (and particularly those listed in Table 1). For instance particles containing HELP, KALA and LAGA are useful.

Particles according to the first aspect of the invention may be targeted to the cells using selective or specific binding agents. The targeted cells often exist within a population of other non-target cells and it is therefore preferred that the means of targeting the particle has at least some specificity towards the target cell over that of the non-target cells.

The binding of the particle to a target cell (referred to herein as primary binding) may be direct or indirect. Antibodies attached to the particle may be usefully employed for directly binding the particle to antigens on the targeted cell. Thus preferred particles may comprise a liposome with a peptide cytolytic agent and an antibody associated with the lipid bilayer.

It is possible to indirectly bind the particle to a cell by having a binding agent which is not directly connected to the particle. For instance an antibody conjugate could be used which has a moiety which will bind to a particle whereas the antibody portion is capable of binding to the target cell.

A variety of other binding agents are known to those skilled in the art and may be used within the scope of this invention. Peptides may be inserted into the lipid bilayer of a vesicle particle which bind to predetermined structures on the target cell. For instance the fibronectin receptor or an integrin may be used to bind to the extracellular matrix of a cell.

It will be appreciated that it will be possible for a single molecule to fulfil the role of binding agent, to be responsive to the metabolic signal and mediate the adaptation of the particle to allow the release, or activation, of the species contained therein.

Thus it is possible to ensure that a particle according to the first aspect of the invention is targeted with high selectivity and/or specificity (e.g. using an antibody) and also that the particle is adapted (e.g. by incorporating a cytolytic agent) such that the incorporated species is activated in response to a predetermined metabolic signal.

The species incorporated within the particle may be any chosen compound. For example the species may be a relatively small molecule such as a dye, electrochemical mediator or receptor agonist. The species may also be a fluorescent molecule (such as latex or polystyrene), an antibody, hormone or an enzyme.

It will be appreciated that the species need not be responsive to the metabolic signal from the cell. While it is within the scope of this invention to incorporate species that may be independently responsive to the metabolic signal, it is an important feature

of this invention that a wide range of species that are not responsive *per se* to the predetermined metabolic signal may be used. Particles according to the first aspect of the present invention are adapted such that the particle is responsive to the metabolic signal by means of molecules on the surface of the particle or by molecules which are easily accessible to the outside environment and ideally the species is held within the particle where it cannot be activated until the particle responds to the metabolic signal. Previously known particles which contain responsive species are unsatisfactory because when the responsive species is inside the particle it is in comparatively poor communication with the predetermined metabolic signal. Whereas responsive species which are either on the surface of known particles or retained within a comparatively permeable known particle are often non-specifically activated.

Particles according to the first aspect of the invention may be further modified such that two types of particle are formed which are capable of aggregating. A first particle may be modified such that a first binding moiety is introduced onto the first particle such that aggregates may form between the first particle and a second particle which has a second binding moiety that is capable of interacting with the first moiety so that aggregates may form as successive particles interact. Such aggregates may contain many thousands of particles and may form a gel like structure around a cell which may even be visible to the naked eye.

Various binding molecules may be used as the first or second moieties to effect secondary binding. Preferred molecules for the first and second moieties are biotin and avidin respectively or biotin and streptavidin respectively. Derivatives and analogues of such moieties may be used. For instance, a protein A-avidin conjugate is useful as antibodies may be bound to the protein A-avidin. Protein A-avidin particles, with antibodies attached, may be prepared in advance of contact with the target cells. Other antibody binding agents may be used (e.g. Protein G).

Particles capable of aggregation represent an important feature of the invention because the aggregated particles may be employed in applications which require high sensitivity. Thus according to a second aspect of the present invention there is provided a method of aggregating a plurality of particles according to the first aspect of the invention comprising providing a first binding moiety on at least some of the particles and at least a second binding moiety on at least some of the remaining particles wherein the first binding moieties interact with the at least second binding moieties such that an aggregate of particles is formed.

According to a third aspect of the present invention there is provided an aggregate comprising a plurality of particles according to the first aspect of the invention wherein a portion of said particles have a first binding moiety and a further portion have a second binding moiety capable of binding with said first binding moiety whereby said particles are aggregated together.

The method of the second aspect of the present invention allows large numbers of particles to be brought into close proximity with a target cells. The said particles can be accumulated proximal to the target cell substantially beyond that normally possible by mono-specific binding between the particles and cells when only the binding agent discussed above is used. The two or more binding moieties are provided on the particle such that particles form aggregates by interaction of the binding moieties (also referred to herein as secondary binding) around those particles which have directly bound to the target cell by way of the binding agent (also referred to herein as primary binding).

The secondary binding need not be specific to the target cells, although it is of clear benefit if the aggregation engendered by use of the secondary binding moieties is itself specific and of high avidity. This benefit arises because, unlike known antibody-targeted particles (e.g. fluorescent particles), the particles of the present invention only respond to a metabolic signal (e.g. pH, redox) from the target cell. Thus any aggregates that may form between the said particles *per se* which do not incorporate a target cell

will not chemically alter and/or release the incorporated substance because the necessary metabolic signal is absent. It will be appreciated that it is possible to arrange the aggregation process such as to minimise the number of aggregates not containing the target cell. One way of achieving this is to treat the cells with the particles under such conditions that few or no aggregates form and then, once primary binding has occurred, a reagent may be added to initiate secondary binding and thereby the formation of aggregates.

When particles modified with avidin and biotin are used secondary binding occurs between the biotin and avidin. The process of aggregating avidin and biotin modified particles may continue until numerous particles form an aggregate particle around the targeted cell. Any secondary binding of the particles to non-target cells and other bodies in the sample will be the result of non-specific binding processes. While in some cases non-specific primary binding may be relatively significant compared to specific binding of particles to target cells in the absence of further aggregative binding of the particles, it is a particularly advantageous that the ratio between the specific and non-specific signals is considerably higher. While there are benefits within the scope of this invention for arranging numerous responsive particles in proximity to the target cell using a secondary process that may not be specific to the target biological cell, it will be understood that it is also within the scope of this invention to arrange a secondary binding process which is also specific to the target biological cell.

When particles further comprise a binding moiety it will be appreciated that the binding agent which mediates primary binding with the target cell need not be directly attached to the particle. For instance an antibody specific to the cell may be conjugated with a binding moiety which will interact with a complementary moiety on the particle. This antibody conjugate then undergoes a primary binding event with the cell and the particles with moieties complementary for binding with the moiety on the antibody conjugate then initiate the aggregation process. Fig. 1 illustrates a preferred aggregation complex in which the primary binding agent is not directly attached to the particle.

It will be understood that additional benefits arise from binding a second population of responsive particles that may incorporate a different species to that incorporated in the first population (e.g. the primary bound particles). By this means, the activation of the species in the first and second population of particles (by the metabolic signal) may allow an interaction between the two species such that a desired reaction occurs. This reaction may be the activation of a prodrug into its active form or may be the formation of a detectable compound (when particles are being used for detection purposes). It will be understood that the probability of the first and second populations coming into close proximity is low except where they both bind to the same target cell. Particularly where specific and avid binding takes place, the probability of such a reaction occurring elsewhere in the medium or sample is correspondingly low, particularly where each population must first be activated in response to the predetermined metabolic signal,. Thus, when two populations of particle are used, the specificity of activation of the particles will be high.

Particles according to the first aspect of the invention which are both targeted to a cell of interest and adapted to be responsive to a metabolic signal may be used for monitoring for the presence of a cell in a sample or may be used in the diagnostics field for identifying particular cells in the body. This kind of use of the particles represents an important feature of the invention and according to a fourth aspect of the invention there is provided a method for detecting cells comprising applying to a sample or subject a particle according to the first aspect of the invention, which incorporates a species that is directly or indirectly detectable when activated in response to the predetermined metabolic signal, and monitoring for the substance.

The method according to the fourth aspect of the invention may be used to detect cells in a sample or subject with great sensitivity and/or specificity. We have found that particles according to the first aspect of the invention may incorporate a substance that is

directly or indirectly detectable which may be used to detect less than 10^4 cells in a sample and as few as 10^1 cells in a sample within a few hours.

Sensitivity may be further improved by using aggregated particles according to the method of the second aspect of the invention.

The method according to the fourth aspect of the invention is particularly useful for the detection of pathogens. Such pathogens may, for example, be in foodstuffs, water samples or even infecting higher organisms. For instance, good food hygiene requires that there is less that 1 pathogenic bacterium (e.g. salmonella or E. coli) in 25 grams of food or within 1 litre of liquid. Conventional monitoring means, which usually require at least 10^4 bacteria, are unable to detect such low levels of contamination. Therefore samples of the food or liquid must be cultured to induce the growth of the bacteria (often with a pre-enrichment growth phase followed by selective growth) in order that there are sufficient numbers to detect. This process may take up to 4 days. The method of the fourth aspect of the invention has the advantage that bacterium may be detected within a much shorter time period, typically within a day and frequently within hours. Such time saving is highly significant for hygiene purposes and for the producer who is able to test and then distribute tested foods more quickly.

Particles used according to the fourth aspect of the invention may release a species directly into the medium surrounding the cell from where it may be detectable (for instance a dye may be released from a liposome in response to a metabolic signal from the targeted cell). Alternatively the species incorporated into the particle may be an enzyme. Such a enzyme may catalyse the formation of a detectable product (the substrate may also be incorporated in the particle, may be in the medium or may even be released from the target cell) in response to the metabolic signal from the cell.

Particles used for the detection of cells need not be in direct contact with the medium containing the cells. When this is the case, the medium containing the cells may

be in communication with a second medium that contains the particles which respond to changes in the medium containing the cells. These changes may be communicated to the second medium by way of a gaseous phase or through a barrier permeable to the activating species (e.g. pH). For example, microbial activity can be detected by capturing in a second medium carbon dioxide produced by metabolic activity of cells in a first medium. The capture of the carbon dioxide in the second medium typically results in a change of conductivity which may be measured. This conductivity change may be amplified in the second medium by incorporating amplifying species in a particle which responds to the pH change in the second medium to release reagents or enzymes (e.g. asparginase) producing a larger conductimetric response. Similarly, the particles may be retained at high concentrations behind a membrane permeable to proton or redox mediating compounds.

A preferred particle for use according to the method of the fourth aspect of the invention incorporates a substance which is an amplifier of the metabolic signal. Such amplifiers make it possible to detect cells in a sample or within a subject undergoing diagnosis with great sensitivity. The particles may be specifically targeted to the cell of interest following which the substance is chemically altered or released such that the metabolic signal which "labels" the cell is amplified for detection.

Typically, the amplifier is retained within the particle such that the amplifier is not detectable prior to the particle responding to the metabolic signal When the particle has responded, the amplifier may be released in a measurable form or may react with other reagents in the medium to produce an indirectly measured analogue of the metabolic signal. In this regard, an important feature of the fourth aspect of the invention is that the amplifier *per se* need not respond directly to the desired metabolic signal and typically does not. Instead, the amplifier is incorporated into a particle whose properties change in direct response to the desired metabolic signal from the targeted cell, the amplifier is released or activated into a measurable form or is allowed to react with other reagents in the medium to form a detectable product.

The amplifier may be a concentrated detectable marker (such as a dye) held inside the particle. Alternatively, the amplifier may be an enzyme or other catalyst. The detectable output from the amplifier in response to the metabolic signal is preferably substantially larger than the signal activating the particle. Thus activation of a particle, such as a sub-micron sized particle containing a dye, by a few tens to a few hundred equivalents may result in the production of at least a few thousand equivalents. In the case of a large molecule such as an enzyme correspondingly fewer molecules may be incorporated in the particle, compared to a lower molecular weight amplifier, an enzyme-catalysed reaction can turnover many times to produce an equivalent or larger effect. Amplifiers for use according to the third aspect of the invention may be selected from a wide range of chemical and biochemical systems, such as inorganics, redox, fluorescent, colourimetrics luminescent or bioluminescent reagents, enzymes or combination thereof producing vapours or gases.

The detected compound may be formed from more than one precursor. When this is the case, it may only be necessary to incorporate one of the precursors involved in the amplifying reaction in the particle. The other precursors may be found in the medium such that they are isolated from the precursor incorporated with the particle until such a time as the particle responds to the metabolic signal. For instance, when the particle is a vesicle containing a peptide cytolytic agent, the metabolic signal may increase the permeability of the lipid bilayer and thereby allow the precursors to interact to form the detectable compound.

Purely by way of example, microbial activity can be indirectly detected carbon dioxide produced by metabolic activity of the cells. An increase in carbon dioxide formation results in a change of conductivity which may be measured. This conductivity change may be amplified by using amplifiers incorporated within a particle which is responsive to the pH change (the metabolic signal). The activated particles may

release an enzyme (e.g. asparginase) which catalyse a reaction to produce a larger conductimetric response.

Examples of amplifiers which may be incorporated into particles for use according to the method of the third aspect of the invention are shown in Table 2.

TABLE 2: AMPLIFIERS

EXAMPLE OF ENCAPSULATED AMPLIFIERS

Encapsulated Amplifier	Example	Detection Mode
Enzyme	Alkaline Phosphatase, β-Galactosidase Glucose Oxidase	Enzyme activity coupled to visual, colourimetric, fluorimetric, luminescent, electrosensing measurement
Co-Factor or Substrate	Coenzyme A,NAD,NADH FAD, ATP	as Above
Fluorphore (self quenching or in combination with quencher)	Calcein, Carboxyfluorescein	Visual, Fluorimetry, Flow cytometry
Chromophore (self quenching) Spin Labels Ions	Arsenazo III Tempocholine Chloride Potassium, Calcium	Visual, Absorbance ESR, EPR Ion-selective electrodes, Dyes

An important feature of the method of the third aspect of the invention is that the particles may be brought into close proximity with the cells such that metabolic activity occurring in the locality of the cell is more directly responsible for the activation of the species. The close proximity of the particle with the cell means that the metabolic signal is not diluted into the medium bulk, thereby increasing the sensitivity of the overall measurement. The activated detectable species is also more directly coupled to the metabolic

activity of the cell, thereby increasing the selectivity and specificity of the overall measurement, towards the cell rather than any similar interfering changes occurring in the bulk medium.

It is not usually practical to increase the proximity between conventional measurement devices and the metabolic changes wrought by cells to minimise the interference and dilution effects apparent in typical media, and particularly those samples containing comparatively small quantities of target cells. In particular, typical samples, such as food and clinical samples, often contain a variety of other non-target cells. Present measurement devices are considerably larger (typically mm to cm dimensions) compared to the cells (typically 0.1 to 50 microns). Thus, particularly in the case where only a few target cells are present in the sample (even when these cells are brought into contact with the measuring device) the measuring device is exposed to large quantities of the sample. Even in the case where the target cells are separated and retained at the measuring device and the bulk of the sample constituents are eliminated, sensitivity at least is not substantially improved for the same reasons.

It is preferred that the method of the fourth aspect of the invention further involves a step by which the particles and targeted cells may be concentrated in a sample. A range of separation methods have been developed to extract and concentrate cells from samples. Many of these methods may be easily modified such that they may be used as techniques for bringing target cells and particles into close proximity. For instance, filtration through membranes designed to retain target cells is widely used for processing water samples containing dilute suspensions and for handling samples of cells in the laboratory. Specific binding proteins, such as antibodies, have also been incorporated onto such membrane filters to retain cells. In both cases, it is possible to co-separate efficiently particles in close proximity to the retained target cells by means of their similar size and their specific binding. Similarly, where such membrane filter or specific binding means are used in conjunction with present measuring devices, it is possible to capture the target biological cells and the

responsive particle in close contact with the measuring device to improve the specificity and sensitivity of measurement.

Similarly, a number of phase partition systems, such as dextran and polyethylene glycol solutions, are used to separate cells. Similarities between target cells and the particles (particularly vesicle particles) render their co-separation relatively straightforward. Again, modification of the phase partition media with agents to improve the selectivity or specificity (e.g. using antibodies) of separation of cells can also be applied straightforwardly to co-separate particles with target cells.

Furthermore magnetic compositions, particularly those modified by specific binding agents such as antibodies (immunomagnetic compositions) are widely used to separate cells from other matter. Magnetic and immunomagnetic compositions may be incorporated into the interior or onto the surfaces of the particles according to the first aspect of the invention to form immunomagnetic particles. Thus cells which have magnetic compositions bound to them and magnetic particles can be co-purified.

Particles can also be easily used with separating techniques such as dielectrophooresis (a means of concentrating cells in an asymmetric electric field). The particles (particularly those comprising a lipid bilayer) possess similar surface and physical properties compared to those of cells and can therefore be co-separated. Similarly, they can be co-transported in a dielectrophoretic travelling wave device. Likewise, cells and particles can be co-captured in an ultrasonic standing wave and can be co-transported in an ultrasonic travelling wave.

Particles can also be used in conjunction with means for isolating cells. Thus the cells may be isolated on solid growth media containing selective growth agents in the media or by selective binding agents (e.g. antibodies). In this case the particles can be incorporated into the solid growth media by introduction of the particles into the liquid media prior to the gelation. The particles may also form a layer (e.g. on the surface of the solid media) or they can be impregnated into the surface of pre-formed solid growth media. Alternatively, the said

particles can be co-isolated with the target cells on the surface of solid growth media, either by placement of mixtures of cells and particles or by contacting the solid growth media with similar mixtures. Similarly, mixtures of cells and particles can be exposed to selective media designed to enrich or select growth or metabolism of the target cells. Individual biological cells can be placed into separate growth components by micromanipulation or by suitable dilution (e.g. into arrays of micro-well plates) such that they are placed in proximity to the said particles.

A preferred way of performing the method according to the fourth aspect of the invention is to utilise particles aggregated according to the third aspect of the invention. For instance when monitoring for the presence of bacteria in a sample, a single bacterial cell may be detected (as a change in colour on a culture plate which is visible to the naked eye about 1 hour after targeting the bacteria) when particles containing enzymes which catalyse a reaction which may be monitored colourimetrically are targeted and aggregated around the bacterium. Hitherto, it has not been possible to resolve a single cell other than by using sophisticated and expensive instruments, such as microscopes and flow cytometers, which require expert use and cannot be applied directly to samples which contain few target cells in a background of a large excess of other bodies in the sample matrix. Therefore, the particles according to the present invention obviate the need for sophisticated instruments for the detection of small numbers of cells. Furthermore the particles serve to increase the specificity and sensitivity of any detection instruments, which those skilled in the field will also understand to mean that the time of detection may also be decreased dramatically using this invention.

The high sensitivity and specificity of methods according to the fourth aspect of the invention means that it is not always necessary to separate the target cells from their original environment and then expose the separated sample to a suitable measuring device. Detectable compounds produced by the responsive particles in proximity to the target cells may be of suitably large magnitude to be resolved by a measuring device, or in the case when amplifiers are used, by the unaided senses of the human body, such as the eye.

Particles according to the first aspect of the invention may also be used according to the method of the fourth aspect of the invention for diagnostic imaging. Purely by way of example, liposome particles may be filled with a substance which is a precursor of a contrast agent (e.g. for CAT) which is also a substrate for an enzyme found in body fluids. The liposome may be targeted to a desired cell type by use of a specific antibody attached to the surface of the liposome. Furthermore the liposome may incorporate a protein cytolytic agent which is responsive to the metabolism of the target cell such that a change in the permeability of the lipid bilayer allows exposure of the substance to the enzyme and results in the formation of the contrast agent. Targeting of the contrast agent using responsive liposomes improves the sensitivity and resolution of various diagnostic imaging methods.

Whereas particles according to the first aspect of the invention may be used in the detection and measurement of cells, the same particles may also be used to treat cells. Thus particles which have been targeted to a cell may respond to a metabolic signal and release bioactive substances for the treatment of the cell. Thus, according to a fifth aspect of the invention there is provided a method of treating cells comprising applying to a cell requiring treatment a particle which incorporates a species which modulates cell activity when activated in response to a predetermined metabolic signal from the cells.

The method of the fifth aspect of the present invention makes it possible to selectively deliver substances which modulate cell activity to target cells. Furthermore, as the substance is released in response to a metabolic signal from the targeted cell, it is possible to achieve high concentrations of a substance which modulates cell activity in the vicinity of the targeted cell.

The method of the fifth aspect of the invention may be used to treat cells such that they may be fortified by that treatment. For instance, to encourage the growth of cultured cells (e.g. by providing a species with growth factor activity to the targeted cell) and especially genetically engineered cells in the biotechnology industry.

Alternatively the particles may contain species which are cytotoxic substances for destruction of the targeted cell. For instance, the particles can be used in the laboratory to destroy an unwanted cell type when it is desired to select between a mixture of cells (e.g. decontaminating a cultured flask of cells which have become fungally infected). Preferably the method of the fifth aspect of the present invention is for the decontamination of a water source by introducing into the water particles incorporating a substance with anti-microbial activity.

The particles may also be used for treating animal or humans. Thus according to a sixth aspect of the present invention there is provided the use of a particle which incorporates a therapeutically effective amount of a species which is activated in response to a predetermined metabolic signal from a cell, for the treatment of medical conditions.

The therapeutically effect species may be a therapeutic substance which is activated by metabolic signals in the locality of a target cell. Such substances are already known (for instance activation of substances by changes in redox chemistry for cancer therapy). However, according to the sixth aspect of the invention, the particle *per se* responds to the metabolic signal. This is advantageous because the use of responsive particles makes it unnecessary for the therapeutic species *per se* to be responsive. Thus a wide range of therapeutic species may be incorporated into the particle, whereas previously only a limited number of responsive substances (which were expensive to manufacture) were available.

The particles may be used to deliver drugs for the treatment of almost any medical condition. However the particles are highly suitable for the delivery of drugs which need to be carefully targeted. For instance the particles are useful for delivering drugs with narrow therapeutic windows (e.g. some forms of chemotherapy) or for the delivery of drugs with serious side effects on none target tissues. Generally the particles are useful when general systemic administration of a drug is undesirable.

The particles are particularly useful for the destruction of cancer cells and may be used to deliver chemotherapeutic agents or radiotherapeutic agents. Cancer cells often possess at least one distinct surface structure suitable for binding the particles and often have a distinct physiology, such as a higher growth rate, often affecting the poise of physiological conditions, such as redox, in their locality. Therefore cancers are most suitably targeted with the particles.

The particles are also useful for treatment of microbial infections, for example a bacterial infection. For instance, an aerosol of particles containing an antibiotic may be inhaled to combat a respiratory infection.

The particles may also be used according to the sixth aspect of the invention in the treatment of other conditions. For instance, cells involved in the immune responses of the body may be treated using particles incorporating therapeutic agents. In some conditions, such as autoimmune diseases, it may be beneficial to suppress particular cells involved in the attack of body tissues, in other conditions, such as poor cellular or immune responses against infections or cancers, it may be beneficial to promote the development, growth or metabolism of particular cell populations or sub-population in order to engender improved protection against such conditions.

The method of the sixth aspect of the invention is also a useful means of treating conditions in which cells found in very low numbers may be harmful to the subject (for instance small and/or newly developed malignant tissues).

The use of more than one type of particle allows the targeting of more than one type of drug to the same site of action. Therefore if two components are required to form the active each could be provided in a different particle. Alternatively several drugs may be delivered to the same target site (e.g. for multi drug resistant infections). It also possible to target many particles to the site of action using a minimal quantity of antibody when different drug bearing particles are aggregated around the target cell. Aggregation of particles also allows

targeting of a greater quantity of less potent or less soluble drug to the site of action. All of the systems described above are activated by the metabolic signal from the target cells thus negating any non specific binding or antibodies to other cells.

Under certain circumstances it is not possible to directly measure a detectable response from particles when normal detection procedures are used, such as non-time resolved measurements of optical output. No matter how large the output is made relative to background, the detectable response may not be able to propagate with adequate magnitude through the sample matrix for accurate measurements (especially when the sample is likely to interfere with measurement). When this is the case, gases and vapours (which are advantageously straightforward to measure) are suitable detectable compounds which may be incorporated in a particle and liberated in response to a metabolic signal. Some gases and vapours may even be detected at low concentrations by the unaided senses of smell and taste. The vapour phase of a liquid or solid sample equilibrates readily with any vapours or gases in the sample which means that gases or vapour are suitable for use with solid samples as well as liquid samples.

Therefore preferred particles according to any aspect of the invention may incorporate a gas or vapour which is capable of being released (or formed) in response to a metabolic signal. It is highly preferred that the particle contains substances which may react to form a gas when the particle responds to the metabolic signal. It is more preferred that the particle contains an enzyme which catalyses the production of a gas from substrate in the media (or substrate also provided with the particle). In the alternative, the particle may contain substrates for an enzyme found in the sample which are converted to a gas when the particle responds to the metabolic signal. Table 3 provides a range of typical chemical and biochemical reactions suitable for the production of gases and vapours following particle activation.

TABLE 3: CHEMICAL AND BIOCHEMICAL REACTIONS SUITABLE FOR THE PRODUCTION OF GAS AND VAPOURS FROM PARTICLES

REACTION	GAS OR VAPOUR PRODUCED
Acetylesterase	Alcohol + Acetate
Acid Phosphatase	Alcohol
Alcohol Oxidase	Aldehyde
Alkaline Phosphatase	Alcohol
S-Alkylcysteine Lyase	Ammonia + Methyl Mercaptan
Amidase	Monocarboxylate + Ammonia
Asparaginase	Ammonia
Arylesterase	Phenol + Acetate
Asides	Nitrogen
Bicarbonate	Carbon Dioxide
Biotinidase	Ammonia
Carbonic Anhydrase	Carbon Dioxide
Carboxylesterase	Alcohol & Carboxylate
Catalase	Oxygen
Catalysts	Various
Cysteine Desulphhydrase	Ammonia + Hydrogen Sulphide
Deaminases	Ammonia
Diamine Oxidase	Aminoaldeyde + Ammonia
Ethanolamine Oxidase	Clycolaldehyde + Ammonia
α-Glucosidase	Alcohol
Glutaminase	Ammonia
Hydrides	Hydrogen
Lactate Oxidase	Acetate + Carbon Dioxide
Metabisulphite	Sulphur Dioxide
Pyruvate Decarboxylase	Aldehyde + Carbon Dioxide
Redox	Various
Serine Dehydratase	Ammonia
Sulphides	Hydrogen Sulphide
Urease	Ammonia + Carbon Dioxide
ß-Ureidopropionase	Ammonia + Carbon Dioxide
Ureidosuccinase	Ammonia + Carbon Dioxide
Explosives	

Gas or vapour liberating particles may be used to detect low levels of pathogenic micro-organisms in food by head space vapour analysis at line. Alternatively, the particles may be left in the food to detect subsequent contamination or growth of micro-organisms by the similar accumulation of the vapour or gas in the head space above the food in the food package. Gases and vapours may also permeate into the packaging material which may be

impregnated with sensing chemicals whose optical or other properties may be read automatically during shipment, shelf storage or on purchase. Alternatively, pungent vapour or bright colours may be produced to warn of a potential hazard in the food.

A further example of a use (according to the sixth aspect of the invention) of gas or vapour liberating particles is for inhalation or ingestion to detect prospective infections in the body (such as the throat/lung or in the gut). This may be used to aid diagnosis of said infection or to investigate the effectiveness of therapy, such as antibiotic treatment. In the latter regard, it is a particular advantage of this invention that it targets the micro-organism and releases vapour in proportion to the relative metabolic signal from the micro-organism. By similar means it is possible to determine the susceptibility of a microbial infection to treatment by a particular antibiotic or other therapy. The ability to determine rapidly the susceptibility of micro-organism to such therapy is of course important prior to or when prescribing treatment, particularly in the light of the increased incidence of multiple drug-resistant strains of micro-organisms. In other infections, such as tuberculosis, it is important to establish the compliance of the patient with such therapy.

The production of gas or vapour by the particles can also lead to the production of sound particularly in those cases where the release of the said gas is comparatively rapid. The sudden production of gas in the particle may then lead to a micro-explosion or bubble release and collapse in the vicinity of the particle. The corresponding mechanical disturbances lead to the production of sound waves which propagate through a dense medium such that they may be detected by a small microphone placed in physical contact with the sample. Particles (for instance bioresponsive antibody targeted liposomes)may be added to a sample such that the aggregate around a target cell. A metabolic signal from the cell causes a response from the liposome complex which is immediately detected by either bulk vapour detection or piezoelectric sound detection (hydrophone). The sound is generated by the metabolic signal form the cell altering the micro-environment around the liposome such that a gas is rapidly generated within the liposomes causing them to burst, or near the liposomes causing bubbles to form which subsequently burst, thus generating a sound signal. This signal is through the

aqueous medium and detected by the hydrophone. Conversely the release of the gas could be detected in the head space above the sample. In this latter case the gas could be generated in the bulk medium by the release of a chemical or enzyme encapsulated in the particles. The use of aggregated particle is important to ensure sufficient sound is generated to be detectable. The main advantage of this system is to detect cells in a bulk medium as a single process. It is possible that the propagation of sound could be detected "in vivo" and "in situ".

It is also possible that the bursting of particles could be used to kill the target cell by either the shock wave (cavitation) and/or by the release of toxic chemical such as a gas, or may serve to introduce other active agents into the cell. The development of a readily detectable signal may also be used to detect analytes other than cells (such as small molecules).

Purely by way of example a sound generating particle may be made by co-encapsulating the enzyme Carbonic anhydrase with sodium bicarbonate inside liposomes. This enzyme rapidly converts bicarbonate to carbon dioxide in the presence of acid. As cells such as bacteria produce acid the pH inside the liposomes will fall when the particle responds to the metabolic signal and carbonic anhydrase starts to produce carbon dioxide. The resultant production of gas will cause the liposome to burst. Other possible methods are the encapsulation of asparaginase and asparagine to produce ammonia or urease and urea to produce ammonia and carbon dioxide.

Embodiments of the present invention will now be described, by way of Examples, with reference to the accompanying drawings.

Figure 1 is a schematic representation of an aggregate of particles according to the invention;

Figure 2 is a graph showing the results of a Mgala assay from Example 2;

Figure 3 is a graph showing the results of a background assay from Example 2;

Figure 4 is a graph showing a glucose oxidase calibration curve, pH 7.1 from Example 2;

Figure 5 is a graph showing a glucose oxidase calibration curve from Example 2;

Figure 6 is a graph of Growth curves of four Salmonella species from Example 3;

Figure 7 is a graph of a Growth curve of S. enteritidis with and without liposomes with a starting innoculus of 2×10^8 cfu/ml from Example 3;

Figure 8 is a graph of a Growth curve for S.enteritidis with and without liposomes with a starting innocula of 2×10^7 cfu/ml from Example 3;

Figure 9 is a graph of a Growth curve for S.enteritidis with and without liposomes with a starting innocula of 2×10^6 cfu/ml from Example 3;and

Figure 10 is a graph of conductometric liposomes control experiment in Example 3.

Fig.1 illustrates a preferred aggregation complex of particles according to the fifth aspect of the invention. In fig 1, antibodies 1 are conjugated to a first binding moiety 2 and make the primary binding interaction with the target cell 3. Particles 4 with a plurality of second binding moieties 5 then aggregate around the conjugate by means of complementary binding between the first 2 and second 5 binding moieties. The aggregate may be enlarged by the addition of further particles with a further binding moieties 6 (which may or may not be the same as binding moiety 2) which the bind to a second binding moiety 5. It will be seen that the size of the potential aggregate will only be limited by the availability of further particles with binding moieties.

EXAMPLE 1

1.1 MANUFACTURE OF BIOTINYLATED LIPOSOMES

1.1.1 Requirements:

Biotin - DPPE	Pierce 22008	10 mg
Glucose oxidase	Sigma G-7141	10,000units
Phosphatidylcholine	Sigma P-3556	100 mg
Dihexadecyl phosphate	Aldrich 27,149-7	10 g
Cholesterol	Sigma C8667	5 g
Sepharose CL6B	Sigma CL-6B-200	500 ml

Econo-column chromatography column 1.5 x 30 cm — Bio-rad 737-1531

10 MM Tris pH 7.1

Plus other equipment used in production of asparaginase liposomes.

Reagent preparation

10 MM tris pH 7.1 Buffer

1.1.2 Method

Weigh 0.0028g dihexadecyl phosphate(DCP) and 0.011g cholesterol into separate scintillation vials, add approximately 5ml of 1:1 chloroform:methanol to each and ensure dissolved (NB DCP may require heat and more methanol to dissolve)
Weigh out 0.040g phosphatidyl choline and dissolve this in approximately 10 ml of 1:1 chloroform : methanol and add this to a round bottom flask along with DCP and cholesterol.
Weigh out 0.0005g of Biotin DPPE and dissolve this in 0.5 ml of 1:1 chloroform : methanol.
Add 0.272 ml of this suspension to the round bottom flask.

[Alternatively add 1 ml of 1:1 chloroform:methanol to the 10ml vial of Biotin DPPE, then add 27.2 µl of this mixture to the round bottom flask and store the rest in freezer.]

Once all the components have been added to the flask, evaporate to dryness on a rotary evaporator with the water bath set at 35°C - this should take less than 1 hour.

Place the flask on the freeze drier to remove the last traces of solvent - leave overnight if necessary.

Dissolve 0.013g of Glucose oxidase (G0D) in 7.5ml of 10M tris pH 7.1.
Add this solution to the dried product in the flask and mix on a rotary shaker for two hours at room temperature.

Remove the stopper from the flask and freeze the lipid mixture in liquid nitrogen whilst gently swirling, until completely frozen. Thaw the mixture in a water bath set at 35°C until completely thawed. Repeat this freeze/thaw process until performed 3 times in total. If necessary the lipid mixture can be stored frozen for months.

Extrusion of liposomes

(Also see instructions for extruder assembly and changing of regulator on N2 cylinder - Lab book (2).)

Assemble extruder with 2 x 0.4μm membranes (for first two passes) or 2 x 0.2μm membranes (for a total of 10 passes, changing the disks 2-3 times if necessary i.e. very slow extrusion).

For extruding
Shut the black valve on the nitrogen line
Close the nitrogen cylinder by turning the handle anticlockwise until locked
Open the green tap
Slowly open the N2 cylinder until the liposomes drip through the plastic tubing into the collecting vessel
When complete, shut off the nitrogen cylinder
Shut off the green tap
Release pressure by opening the black valve
Release line pressure by opening the green valve
Shut both green and black valves, reload liposomes and start again
Store liposomes in fridge prior to purification

Purification of liposomes by gel filtration

Purification of the biotin liposomes is done by gel filtration using a CL6B column. The filtration is done by gravity and takes about 1 hour per run. The column volume should be approximately 53 mls with a void volume ranging from 10 - 14 mls (this must be measured for each new column on first use with liposomes).

Pouring the column
Pour ~50ml of CL6B into a beaker and add 10-20ml Tris buffer pH 7.1. Mix and pour this mixture into the column in one go. There must be no gaps in the column or cavitation may occur.

Wash the column through with 50 ml volume of tris buffer pH 7.1.

NB The column must not dry out at any time.

Take the liquid level to just above the top of the column. Load 1ml of liposomes and start to measure the elluent. Take the liquid level to the top of the column again then add buffer. You should get around 10-15ml of clear buffer before the liposomes start to come through and the solution will turn cloudy. The column of liquid that comes through before the liposomes is the void volume. With 1ml of liposomes you should get 4-5 0.5ml fractions, number these accordingly. When the liposomes have come through flush the column with 50ml volume of buffer. If the column is not to be used for several days, dissolve a few crystals of sodium azide in 50ml buffer and pass this partially through the column. Before using again discant azide containing buffer and flush through 50ml volume of the fresh buffer to remove the sodium azide solution. The column can be left for several months with sodium azide in. Store the liposomes in the fridge prior to assaying them or further purification.

Assay of liposomes

The purified biotinylated liposomes are assayed on a spectrophotometer to determine the level of background activity prior to use. If background levels are too high further purification can be done using trypsinised beads (see protocol for asparaginase liposomes, Lab book 1).

Requirements

D-Glucose		
Horseradish Peroxidase (HRP)	25mg	Sigma P6782
5-amino salicylic acid	25g	Sigma A3537

50MM sodium phosphate buffer pH 6.

Prepare two solutions, A and B as follows:

A: 0.3g of D-glucose
200 IU of HRP

HRP 110 units/mg solid
Therefore for 200 units use 1.8mg = 0.0018g
(can store as stock for ~ 1 month)

Dissolve both in 4ml of phosphate buffer pH6. Will store in fridge for ~ 5 days.

B: 0.005g of 5-amino salicylic acid dissolved in 5ml phosphate buffer pH 6.
Wrap in silver foil as it is light sensitive.

Set up a UV Spec at 450nM wavelength. Use a 1.5ml cuvette and zero the machine against a solution with 0.5ml solution A and 0.5ml solution B. Once a baseline has been established, add 30µl of a liposome fraction, put parafilm over the cuvette and invert to mix then return to the spectrophotmeter. Allow to run for 5-10 mins so that an assessment can be made of the background activity of the liposomes.

Then to the same cuvette add 10µl of triton, mix as before and return to the spec for a further 5-10 mins. An increase in absorbence should be seen indicating a release of the enzyme which will react with the HRP in the presence of glucose to produce a red/brown colour.

Print off the graphs, label with the batch number of the liposomes and the fraction number. Repeat for all fractions obtained from the gel filtration.

All fractions that show a low background with a significant increase in absorbence on lysis, are acceptable and can be used for further experimentation. These fractions can be pooled and assayed again as a final check. All fractions in which activity is considered to be too low should be discarded.

If background activity is unacceptable carry out a further purification step using trypsin beads.

Aggregation experiment

This experiment is designed to confirm the presence of biotin on the liposomes by aggregating the liposomes with avidin. Addition of a sufficient amount of avidin causes the liposomes to clump up (increasing absorbence) and fall out of suspension, forming a visible precipitate and eventually showing a decrease in absorbence.

Method

Warm up the spectrophotmeter at 600nm. Zero the machine against 1ml. Tris buffer pH7.1. In another cuvette add 0.9ml of buffer with 100µl of purified biotinylated liposomes. Run in the spec (a relatively flat line should result indicating the turbidity of the liposomes).

Make up a solution of 1mg/ml Avidin (solution C). [C: 0.001g Avidin in demin.] Add 60µl of solution C to the cuvette, mix and return to the spec. A dramatic increase in absorbence should be seen indicating aggregation of the liposomes which in turn scatter more light. Allow this response to level off then add a further 60µl of solution C - a similar, though less dramatic, effect should be seen. Repeat the addition of the avidin solution until a decrease in response is seen. This indicates avidin saturation whereby the liposomes no longer aggregate and further addition of avidin dilutes the observed turbidity . On standing the aggregated liposomes start to fall out of solution and their light scattering ability is reduced.

Before carrying out further experiments with the liposomes, they must be made pH sensitive by the addition of M-gala (see protocol).

Production of Agar Plates

Requirements
Agar recipe for liposome Agar Base (LAB)

Peptone	5g
Yeast Extract	2g
Glucose	10g
Agar	15g

Weigh out the ingredients and add 1 litre of water, then adjust pH to 8 prior to sterilisation dispensing in 80ml volumes and autoclave at 121°C ± 1°C for 15 mins. The following supplements are added to the agar after sterilisation before pouring the plates (i.e. added at 50°C).

Make a solution of 1mg/ml 5-amino-salicylic acid. [0.001g 5-amino-salicylic acid in demin, filter sterilised.]

Make a solution of HRP at 100IU/ml in demin and filter sterilise.

To a 80ml bottle of agar at 50oC add 20ml of the 5-amino-salicylic acid solution and 100ul of the HRP solution. "Pour" the plates in 20ml volumes using a sterile disposable pipette.

When set, dry the plates as normal. If the plates are to be stored for a few days store them in a black plastic bag to exclude light.

Check the pH of the set agar to ensure it is above pH6.8.

Mechanism of Action
The liposome contain the enzyme glucose oxidase and the peptide m-gala which is pH sensitive (see protocol for the addition of M-gala to the liposomes). In the presence of acid - added to the plate or produced by growing organisms - the pH close to the liposomes decreases, the m-gala changes conformation and releases the GOD into the media. In the presence of glucose (in the agar plate) the GOD produces H2O2 which reacts with the HRP and the 5 amino salicylic acid (in the agar plate) to produce a brown colour. Alternatively, as a control, the liposomes can be lysed using a detergent and again the colour change is seen.

Bacterial Detection

Requirements

Avidin	Sigma A9390	10mg

CSA Antibody	Bactrace 01-91-99	5mg
Protein A-Biotin	Sigma P216S	4mg
Biopur tips	BDH 307/8513/07	

Sterile phosphate buffered saline (PBS) pH 7.4.
M-gala (desalted & unpurified). Peptide products Ltd. 10 mg
[Peptide Products Ltd
Saddleworth Business Centre
Huddersfield Road
Delph, Saddleworth
Oldham. OL3 5DF
Tel: 01457 875798
Fax: 01457 871088
NB £870 + VAT + £10 delivery for 10mg.

For bacterial detection to work the liposomes need to be made pH sensitive by the addition of M-gala. As M-gala will lyse the liposomes at pH <6.5, all proceeding assays must take place at pH's higher than this.

Addition of M-gala

Assay
Re-assay the liposomes on the spec at 450 nm using the following procedure.

0.25ml solution A + 0.25ml solution B + 0.5ml 10MM sodium phosphate buffer pH 8.2.
(This should give a pH of ~ 7.15).
Assay the pooled liposomes as before (i.e. zero background & lysis) using 100 µl of liposomes.

A reduced enzyme activity slope should be seen due to the reduced amounts of substrate and non optimal pH for enzyme. The assay time may need to be increased from the previous 10 mins.

M-gala addition
Filter sterilise remaining liposomes.
Filter sterile a few mls of 10MM sodium phosphate buffer pH 8.2
Make solution D: 0.1mg/ml M-gala in demin and filter sterilise. Store up to 1 month 4°C (D: 0.0001g M-gala in demin).

Add in the following order
0.5ml sterilised liposomes
0.5ml 10MM sodium phosphate buffer pH 8.2 sterile
0.5ml sterilised solution D

Incubate at room temperature 30 mins

Bacterial Detection Method

Grow a Salmonella culture in TSB overnight at 37°C. In the morning sub the culture with fresh TSB and grow for a further 2 hours (so Salmonella culture is in an active growth phase).

After 2 hrs dilute the culture in MRD to a -8 dilution. Enumerate these dilutions on non-selective agar plates (PCA).

Dissolve 1mg CSA antibody and 1.3mg Avidin in 833 microlitres of sterile PBS then filter sterilise through a 0.22um filter. Then add 167 microlitres of 1mg/ml protein-A-Biotin (also filter sterilised) in PBS and incubate for 30 mins. Add 200 microlitres of this antibody-protein A-Biotin-Avidin to 1ml of bacteria and incubate for 90 mins (use 1ml of each dilution).

Spin this down at 13,000 rpm for 10 mins, discard the supernatant, resuspend the pellet in 1ml PBS, spin down again and discard the supernatant again. Add 100µl of biotin liposomes + 900µl of PBS to the mixture and incubate for 30 mins prior to spinning, discarding the supernatant, washing with PBS, respinning and discarding the supernatant. Resuspend the pellet in 20µl of sterile PBS and add this to the dried agar plate. Incubate the plate at 37°C and take photos at regular intervals.

NB The first stages of validation at LGC will involve validation with pure cultures of Salmonella and no antibody, i.e. to assess sensitivity and time to detection alone.
Plate out the Salmonella dilution cultures

EXAMPLE 2

2.1 pH METHODOLOGY FOR AGAR PLATE ASSAY.

2.1.1 Manufacture of biotinylated liposomes - Version one.

Requirements.
Biotin DPPE (Pierce 22008), Glucose oxidase (Sigma G-7141), Phosphatidylcholine (Sigma P-3556), Dihexadecyl Phosphate (Aldrich 27,149-7), Cholesterol (Sigma C8667), Sepharose CL-6B (Sigma CL-6B-200), Econo-column chromatography column 1.5 x 30cm (Bio-Rad 737-1531), 10mM Tris pH 7.1 plus equipment used in the preparation of asparaginase liposomes.

Procedure.
Weigh out the following; Phosphatidyl choline 40mg, Cholesterol 11mg, Dihexadecyl phosphate 2.8mg and dissolve in 5ml 1:1 Chloroform:methanol, add to a round bottom

flask. Weigh 0.272mg Biotin-DPPE made up as 1mg/ml 1:1 chloroform:methanol, mix in the flask and evaporate to dryness on a rotary evaporator. Place on a freeze-drier to remov last traces of solvent. Hydrate with 13mg of glucose oxidase in 7.5ml Tris buffer pH 7.1. Continue to produce liposomes as for asparaginase liposomes but do not trypsinise. Purify liposomes by gel filtration on a sepharose CL-6B column using 10mM Tris pH7.1 and store in the fridge.

Version two of this protocol involved leaving the enzyme in contact with the lipid mixture overnight in the fridge before extrusion of the liposomes, storage of the liposomes at 22°C after extrusion and at all steps thereafter, and assaying each fraction of liposomes from the CL-6B column, so that any liposomes with unacceptable background levels can be discarded before pooling. 30ul of liposomes only were found to be necessary for the spectrophotometer assay. A further experiment was also designed to confirm the presence of biotin on the outside of the liposomes - the method for which follows;

Avidin aggregation.
This experiment confirms the presence of biotin on the outside of the liposomes by aggregating the liposomes with avidin. Addition of a sufficient amount of avidin causes the liposomes to clump up (increasing the absorbence) and, with the addition of more avidin they will fall out of suspension (hence decreasing absorbence).
Using the spec at 600nm the machine is zeroed against 1ml Tris buffer pH 7.1. Add 100ul of purified biotinylated liposomes and a relatively flat line should be seen indicating the turbidity of the liposomes. 60ul volumes of 1mg/ml Avidin are added to the spec until the absorbence begins to decrease indicating avidin saturation whereby the liposomes no longer aggregate and further addition of avidin dilutes the observed turbidity.

2.1.2 Assay of liposomes.

Requirements.
D-glucose, Horseradish Peroxidase (HRP, Sigma P6782), 5-Amino-salicylic acid (5-ASA, Sigma A3537), 50mM Sodium Phosphate buffer pH6.
Procedure.
Prepare two solutions;
A: 50mg D-glucose in 20mls phosphate buffer pH6 plus 100IU/ml HRP in Phosphate buffer pH6.
B: 1mg/ml 5-ASA in Phosphate buffer pH6.
Assay 0.5ml of A and 0.5ml B in a UV/Visible spectrophotometer at 450nm. Zero the machine against A and B then add 100ul of liposomes.

2.1.3 Mode of action
Biotinylated liposomes are made and M-gala added to make them pH sensitive. The capture complex is formed when the target organism is captured by the Antibody-Protein A-Biotin-Avidin. When the liposomes come into contact with the capture complex, the biotinylated liposomes attach to the avidin. The whole complex is then dropped onto an agar plate containing glucose and HRP. As the target organism grows it lowers the pH of the media in

the immediate surroundings which in turn lyses the liposomes releasing the GOD. The following reaction then occurs;

GOD + (Liposomes)	Glucose (media)	H_2O_2
H_2O_2 +	HRP +5ASA (media) (media)	Colour

Hence, a brown colour developing on the medium is seen in response to the presence of viable target organisms on the plate. The specificity of the antibody can be utilised by a number of spin wash steps to remove non-target cells or alternative capture mechanisms can be employed.

2.1.4. Control experiments

Control experiments were carried out on the agar plates to ensure that;
(1) The liposomes were pH sensitive.
(2) The liposomes contained sufficient GOD to produce a visible colour response.
(3) The liposomes showed a sufficiently low background colour development for the positive signal to be evident.
Each of these controls were carried out as follows.
(1) The pH control assay was carried out by dropping a 25ul volume of M-gala liposomes onto a pH6 LAB plate (see 2.1.6). A colour response should be seen within 10 minutes.
(2) 25ul volume of M-gala liposomes were dropped onto a pH 7 plate then 10ul of triton was added. The detergent lysed the liposomes releasing the GOD and an almost immediate colour development was seen.
(3) The liposome background assays are important control experiments as their colour development is compared to positive experimental signal colour . Colour stronger than the control at any given point is considered a positive result, whereas colour the same as or less than the control is considered negative.
In the first experiments this control generally consisted of 25ul M-gala liposomes. However, as the experiments consisted of 25ul m-gala liposomes diluted with 20ul culture, these controls were in fact more concentrated and therefore likely to produce more colour. The next controls used therefore consisted of M-gala liposomes made up to the same volume as the experiments with 10mM sodium phosphate buffer pH8.2. However, the best control used were found to be killed Salmonella cells taken through the protocol as the experiments themselves (see section 2.1.6). On the first instance formalin was used to kill the cells, though later heat killed cells were found to give more realistic results.

2.1.5 Addition of M-gala.

For bacterial detection work the liposomes need to be made pH sensitive by the addition of M-gala.
Filter sterilise the remaining liposomes and filter sterilise a few mls of 10mM sodium phosphate buffer pH8.2. Make a solution of 0.1mg/ml M-gala in demin adding a few

crystals of ammonium carbonate to dissolve, and filter sterilise. Add 500ul sterilised liposomes, 500ul 10mM sodium phosphate buffer and 500ul sterilised M-gala solution (or proportions thereof). Incubate at room temperature 30mins.
Reassay the M-gala-biotinylated liposomes as before (above) using 250ul A, 250ul B and 500ul sodium phosphate buffer pH8.2 and add 200ul liposomes.

2.1.6 Plate Assay.

Media preparation
Agar media is prepared in the usual way to the following recipe:

Peptone	5g/L
Yeast extract	2g/L
Glucose	10g/L
Agar	15g/L

Prior to pouring the plates filter sterilise 1mg/ml 5-ASA to add 20% to final media volume. In 10ml plates add 2ml of 5ASA and 20ul of 100IU/ml HRP. When poured and set check the pH of the plates to ensure that it is above 6.8.
Version two of the protocol adds the 5-ASA to the media prior to autoclaving as this produces agar plates with a more uniform pH across the surface. If pH 6 plates are require the media should be taken to pH 7 before autoclaving, for pH 7 plates take the pH to 8.3 before autoclaving.

Plate assays were carried out according to the outline in 2.2.1. Large antibiotic assay plates were used which enabled 49 drop tests to be carried out per plate. The drop format was used in the first instance as this allowed for a concentrated colour development to be seen clearly. Spreading the liposome-bacteria complex across the plate was also tested.

Bacterial detection.
Requirements.
Avidin (sigma A9390), CSA Antibody (Bactrace 01-91-99), Protein A-Biotin (Sigma P2165), PBS pH 7.4.
Version two of the protocol introduces the possibility of using Protein G (Sigma P8045) in place of Protein A as its binding to the antibody may be better. For this 250ul of protein G is used in place of 167ul protein A.

Procedure.
A Salmonella culture is grown in TSB(Oxoid CM129) or BHI (Oxoid CM225) media overnight at 37oC. In the morning it is sub-cultured into fresh media and allowed to grow for a further 2 hours - this ensures that the culture is in the log phase of growth for the experiment. Serial dilution of the culture is performed in PBS.
Dissolve 1mg CSA antibody and 1.3mg Avidin in 833ul sterile PBS. Then add 167ul of 1mg/ml Protein A-Biotin in PBS and incubate for 30mins. Add 200ul of this Antibody Protein A-Biotin Avidin complex to 1ml of bacterial dilution and incubate for 90mins.

Spin this mixture down at 13,000rpm for 10mins, discard the supernatant, resuspend the pellet in 1ml PBS, spin down again and discard the supernatant. 100ul of biotin liposomes (pre-incubated for 30 mins with 0.1mg/ml Mgala in demin) and 900ul of PBS was then added and the mixture incubated for a further 30 mins prior to spinning, discarding, washing with PBS, respinning and discarding the supernatant. The pellet is then resuspended in 20ul PBS and this volume is then added to the dried agar plate. The plate is incubated at 37oC and observed at hourly intervals up to approx. 6 hours.

Version two.
Version two includes a number of additional filter sterilisation steps as either the antibody of avidin were found to be introducing contamination in earlier experiments.
The first supernatant from the spin-wash steps was kept aside and enumerated in order to calculate the ratio of bound to unbound cells using each antibody.
Following problems with high background signals from the liposomes, the amount of liposomes per experiment was reduced from 100ul to 25ul.
An additional incubation step with avidin was included prior to the final spin wash step, i.e. after addition of the liposomes, as this enabled aggregation of the liposomes hence giving a stronger signal per organism captured. A volume of 12ul was added.

2.1.7 Calibration curves.
Calibration curves were plotted to determine the relationship between absorbence at 450nm and GOD activity in IU's. This was done by assaying increasingly greater concentrations of GOD in equal volumes of solutions A and B made either in pH 6 or pH 7. These graphs were then used later to determine the encapsulated GOD activity in each batch of liposomes allowing for a greater batch to batch control and comparison of the liposomes.

2.1.8 Sensitivity Tests.
These experiments were designed to assess the sensitivity and time to detection of the pH sensitive liposomes on agar plates when no antibody specificity steps were introduced. Once the antibody separation step is introduced, issues of antibody specificity and loss of liposomes by wash steps become important. These initial sensitivity tests allowed for the sensitivity of the liposomes alone to be tested. The method involved growing up overnight cultures of the target organisms, diluting them in PBS to a -8 dilution then adding 10ul of each dilution onto the agar plate along with 25ul of M-gala liposomes. Growth of the bacteria changed the pH of the medium causing lysis of the pH sensitive liposomes and hence a direct colour response, though as the liposomes and bacteria were not necessarily in close proximity, because of the absence of the antibody binding step, it was expected that the response seen would be slower then the antibody assay though faster than standard overnight incubation and visualisation of the colonies.

2.1.9 Antibody Sensitivity Test.

These assay involved the whole assay as specified in 2.2.5. Bacterial dilution series were utilised as above (2.2.7) and 20ul drops of the bacteria-Antibody-Liposomes complex were dropped onto the plate.

2.1.10 Specificity Tests.
Specificity tests were carried out using the Antibody binding protocol with a range of non-target organisms. In the first instance it was hoped that the spin wash steps would be sufficient to remove non-target organisms from the bacteria liposome complex, though later further developments had to be introduced in order to fully utilise the specificity of the Antibody, such as spread plating to ensure any non-target colonies were physically distant from the liposome.

2.2 RESULTS

2.2.1. Liposome Quality Control. data.
Throughout the pH agar plate assay development and evaluation, many different batches of liposomes were prepared, often each batch being prepared by a slightly modified protocol. For the method evaluation work, in order to have some standardisation of criteria between experiments, a number of control experiments were devised and limits set so that the quality of liposomes between experiments could be compared. The control experiments used are outlined in section 2.1.4 and a typical set of results are as follows.
(1) pH control assay.
25ul of M-gala biotinylated liposomes was dropped onto a pH6 agar plate (BN5 in photograph) and this showed a colour response in approximately 10 minutes if the lipsosomes were pH sensitive, a slower and less clear colour development should be seen on a pH7 plate (BN4 in photograph).

(2) As long as colour development was seen on a pH6 agar plate this demonstrated the presence of sufficient GOD for a visible signal. In addition, liposomes with triton was always included as a control.

(3) Liposome background assay.
A variety of methods were used in order to create a realistic background control assay, as outlined in 2.1.4. Again each of these were run with every experiment and typical results are seen in 3.2.3. and 3.2.4. In addition to a plate assay, a spectrophotometric assay was carried out. This enables a value in absorbence units per minutes to be calculated on both the background and lysis rates, and after data from several batches had been obtained, numerical limits were set as maximum and minimum figures.

A typical result of such an assay (method section - Assay of liposomes) is shown in Figure 2.From the slope of the line during the liposome background and lysis section of the graphs, calculations of the rate in A/min could be made.

A maximum background rate of 0.03 A/min was decided on with a minimum background:lysis ratio of 1:10. Few batches of liposomes fell outside of these limits once the complete purification protocol was used, and all batches within these limits were shown to give acceptable colour development.

For Fig 2 calculations;
Background 0.014 A/min
Lysis 0.162 A/min
Ratio background to lysis x11.
Encapsulated GOD activity 0.193IU.

Further work on optimising the liposome production procedure was started, and preliminary results suggested that liposome quality can be increased. In the original protocol the lipid mixture was rehydrated with GOD for 2 hours at room temperature after freeze-drying. By leaving the GOD in contact with the lipid mix overnight at refrigeration temperatures, background to lysis ratios of over 100 times were achieved with a related GOD encapsulation figure of 0.64 IU (Fig 3), when compared with about 0.3 IU using the 2 hour rehydration step.

2.2.2. Calibration curves.

Although the setting of QC limits for batches of liposomes was very helpful, the figures as they were, did not give any quantitative values of the amount of enzyme available for the reaction to take place. In order to calculate this, an enzyme calibration curve was established to compare absorbence at 450nm to concentration of GOD in IU. The calibration curve for the pH7.1 assay only is shown as Fig 4.From this graph maximum rates were taken for each concentration and again plotted against the concentration of GOD in IU (Fig 5), and a line of best fit established.

From the equation of the line of best fit (Fig 5), a calculation was performed using the maximum rate of change of absorbence during lysis, and hence a figure could be given for the encapsulated GOD activity of the liposomes.
e.g. for Fig 6, lysis rate = 0.162 A/min.

Encapsulated GOD activity $x = \frac{y-0.0198}{0.7375}$

x = IU
y = A/min

x = 0.193IU GOD

2.2.3. Sensitivity test.

This experiment looked at the ability of the pH sensitivity liposomes to detect bacteria on agar plates per se, that is without the antibody and any of the spin wash steps designed to introduce specificity. Again this test was carried out several times, though one experiment only is given here.

Plate plan

S.typh. 25µl -1	S.typh. 25µl -2	S.typh. 25µl -3	S.typh. 25µl -4	S.typh. 25µl -5	S.typh. 25µl -6	S.typh. 25µl -7
S.typh. 25µl -1 + lips	S.typh. 25µl -2 + lips	S.typh. 25µl -3 + lips	S.typh. 25µl -4 + lips	S.typh. 25µl -5 + lips	S.typh. 25µl -6 + lips	S.typh. 25µl -7 + lips
L.mono. 25µl -1	L. mono. 25µl -2	L.mono. 25µl -3	L.mono. 25µl -4	L.mono. 25µl -5	L.mono. 25µl -6	L.mono. 25µl -7
L.mono. 25µl -1 + lips	L. mono. 25µl -2 + lips	L.mono. 25µl -3 + lips	L.mono. 25µl -4 + lips	L.mono. 25µl -5 + lips	L.mono. 25µl -6 + lips	L.mono. 25µl -7 + lips
S.typh. 25µl -8	S.typh. 25µl -8 + lips	L.mono 25µl -8	L.mono 25µl -8 + lips	Lips in buffer control	Lips + M-gala 25µl control	Lips + M-gala 25µl control
S. ent. 25µl -1 + lips	S.ent. 25µl -2 + lips	S.ent. 25µl -3 + lips	S.ent. 25µl -4 +lips	S.ent. 25µl -5 +lips	Media control	PBS control
S.ent. 25µl -6 + lips	S.ent. 25µl -7 +lips	S.ent. 25µl -8 +lips	E.coli + lips	S.aureus + lips	Media control	Lips + triton

Comparing the colour development of the experiments to the controls (E6 and E7) and relating the positive colour development to cell counts gave the following results for this particular experiment.

S.typhimurum positive at 6×10^7 cfu/ml (-1 dilution) at 5 hours 20 mins.
L.monocytogenes positive at 7×10^7 cfu/ml (-1 dilution) at 5 hours 20 mins *S.enteritidis* positive at 5×10^7 cfu/ml (-1 dilution) at 5 hours 20 mins.

Repeat experiments gave positive results at 4 to 5 hours with 10^4-10^6 cfu/ml. Standard overnight incubation of plates without liposomes would generally give visible colonies in 16-18 hours, therefore these experiments are already demonstrating the potential of this method by improving time to detection by more than 10 hours, making detection within one working day on an agar plate a possibility.

2.2.4. Antibody sensitivity tests.

By introducing the antibody binding step and hence bringing the liposomes into close proximity with the bacteria, it was thought that the time to detection for this method could be greatly improved. One experiment only is shown here again.

Plate plan

S.ent. -1 + lip	S.ent. -2 + lip	S.ent. -3 + lip	S.ent. -4 + lip	S.ent. -5 + lip	S.ent. -6 + lip	S.ent. -7 + lip
S.typh. -1 + lips	S.typh. -2 + lips	S.typh. -3 + lips	S.typh. -4 + lips	S.typh. -5 + lips	S.typh. -6 + lips	S.typh. -7 + lips
S.ent. -8 + lip	S. typh. -8 +lip	S.ent. 24 hours -6 + lip	S.typh. 24 hours -6 + lip	S. ent formalin + lip	S.typh. Formalin + lip	
S.ent. 20min heat + lip	S.ent. 30min heat + lip	S.typh. 20min heat + lip	S.ent. 30min heat + lip	Lips + buffer control		
C.freundii + lip	E.coli +lip			B.cereus + lip		
S.ent. -5	S.ent. -6	S.ent. -7	S.ent. -8	S.ent 24 hours -6		
S.typh. -5	S.typh. -6	S.typh. -7	S.typh. -8	S.typh 24 hours -6		Lips + triton

This experiment compared heat and formalin killed cells as controls, the heat killed cells (D1-D4) were used as control comparisons. Again comparing colour development of experiments with controls and relating these to cell counts, the following results were achieved.

S.enteritidis 185 cells positive in 30 mins
18 cells positive in 2 hours

S.typhimurum 115 cells positive in 1 hour
11 cells positive in 2 hours

Repeat experiments gave comparable results with about 2 hour detection of 10^1-10^2 colony forming units. The inclusion of the antibody binding step therefore greatly improved time to detection, thus making detection in a single working day a realistic proposition from a culture containing 10^2 or more viable growing organisms.

Although these results were achieved on the agar plate in about 2 hours, the preparation time using the assay in its present form is considerable (approximately 7 hours). However, this is because the protocol at present is tailored to research , allowing monitoring at each step, requiring the bacteria, antibody, protein A, avidin, liposome complex to be built up step by step with incubation periods. It would be a relatively straightforward procedure to supply at least the antibody-protein A-avidin complex complete, hence requiring only incubation with the bacteria and then the liposomes. Such issues should not, however, distract from the significance of the results achieved here as detection of 100 cells in an hour on an agar plate is a remarkable achievement in itself.

For the first few antibody binding experiments, the first wash supernatants were enumerated after the antibody binding step in order to assess the percentage of target organisms bound by the antibody. This was done so that if there was any lack of sensitivity with the methodology, this could be attributed to either the liposomes method or the antibody binding. In fact, the antibody appeared to bind greater than 95% of the target organisms on each occasion when both the Salmonella antibody and Listeria antibody were used with the spin wash protocol, and this percentage binding was even maintained at the lower innocula levels.

2.2.5 Specificity tests .

During the antibody sensitivity test, a number of negative control organisms were also run. These were generally run with high innocula concentrations (10^7-10^9 cfu/ml), so as such were a worse case scenario. The following organisms were tested;

E.coli; two negatives and one positive result.
S.aureus; one negative and one positive result.
L.monocytogenes; two positive results.
C.freundii; one negative result.
B.cereus; one positive result.

These results were all produced with the CSA antibody and using spin wash steps only as a means of separation of target and non-target organisms. At 13,000 rpm both antibody/bacteria complexes and free bacteria would be found in the pellet. It is therefore no surprise that the negative control organisms were carried right through the preparation steps and gave positive results. The variability of results is likely to be caused by the different innocula and different growth rates and hence acid production by the organisms on this specific media. Although each of these organisms can utilise glucose as a growth media and hence will produce acid, other factors such as utilisation of peptone (which produces alkaline biproducts) may be having an effect. It is therefore apparent from these experiments that additional specificity steps are required. One possibility was to go through the sample preparation as before though spreading the resulting mixture over the plate rather than performing a spot test. In theory this would separate target organisms

surrounded by liposomes from non-target organisms with no associated liposomes. As the target organism grew the cells would produce a localised pH change, lysing the liposomes and a colour change would be seen around the vicinity of the target colony. Around the non-target colony there would be no liposomes (assuming that all available liposomes were bound to the Protein/antibody complex) and hence any growth and resulting localised pH change would not produce a colour response. A single experiment has been carried out to investigate this at present, and preliminary results suggest that spreading alone is not sufficient to achieve the required specificity. Instead of specific localised colour development, a general spreading of brown colour was seen with both dilution cultures of Salmonella or mixed Salmonella and E.coli cultures.

Other options are still available to improve the specificity of this test such as using a solid phase capture step, using a magnetic separation step or, more simply, using the aggregated liposomes complex to pull the target organism out of suspension and away from the non-target organisms.

2.2.6 Viability assessments

One advantage of this technique is that a positive result relies on the growth of the target organisms and so only viable cells will be detected. One question that needed addressing was whether the growth phase of the organism had any affect on the time to detection for this method. The assumption was made that the cells would have to be in an active growth phase in order to give rapid results, so the sensitivity tests reported here were carried out on fresh 2 hour sub-cultures from overnight cultures. For a brief assessment of viability to be made 2 hour, 24 hour, stressed and killed cells were compared. A summary of results from a number of experiments follows;

S.enteritidis.

	Cell count	Result
2 hour sub-culture	10^3	Colour 30min-2 hours
24 hour	10^3	no colour
Stressed 55°C/2 hours	10	colour in time comparable with 2 hour cultures.
20 min heat kill	0	minimal background colour 4-6 hours
30 min heat kill	0	minimal background colour 4-6 hours

S.typhimurim

	Cell count	Result
2 hour sub-culture	10^2	Colour 1-2 hours
24 hour	10^2	no colour
20 min heat kill	0	minimal background colour 2 hours
30 min heat kill	0	minimal background colour 4-6 hours

These result suggest that the cells do in fact need to be in an active growth phase in order to give a rapid positive results. However, the fact that the 24 hour old cultures did not give a positive results even overnight is somewhat curious, as at least a background colour would be expected. The S.enteritidis cells stressed at 55°C for 2 hours gave results in a timescale comparable to the 2 hour culture, and again this does not quite match with the other results - again demonstrating the need for further experimentation. These results point to the necessity for further work in this area, and such work should be linked in to the introduction of a recovery step prior to the liposome assay itself.

EXAMPLE 3

3.1.1 Preparation of Asparaginase liposomes.

Requirements.

Phosphatidylcholine (Sigma P3556), Cholestrol (Sigma C8667), Dicetyl Phosphate (Sigma D2631), Asparaginase (1000IU/ml, CAMR), 50ml Quickfit 24/29 round bottom flask, Chloroform (Analar), Methanol (Analar), Liquid nitrogen, Waterbath at 35°C, flask shaker, extruder attached to oxygen free nitrogen line, 0.4 and 0.2 micron filters (nucleopore polycarbonate) and drain discs for extruder, 100mM Tris pH 7, 0.22 micron sterile filter units (millipore).

Procedure.

The asparaginase should be prepared by diluting the freeze-dried contents of a 10,000 unit vial in 0.22 sterile demin to 1,000 units per ml and stored in the freezer in 1ml aliquots until required.
The phosphatidyl choline should be made up as 50mg/ml in a 1:1 mixture of chloroform:methanol, this is stored in the freezer prior to use. Remove 800ul of this mixture and put in a 50ml round bottom flask. Weigh out 11mg of cholestrol and dissolve in 2ml chloroform :methanol and add this to the round bottom flask. Weigh out 2.8mg of dicetyl phosphate and dissolve in chloroform:methanol and also add to the round bottom flask.
Rotary evaporate the sample using a water bath set at 35°C until all the solvent has been removed leaving behind a thin lipid film on the flask. The flask is then placed on a freeze-drier overnight to ensure that all last traces of solvent are removed.
Add 6ml demin water, 750ul 100mM Tris pH7 and 750ul asparaginase (1,000IU/ml) to the flask and mix gently for 2 hours at room temperature. After this time freeze the lipid mixture in liquid nitrogen until completely frozen. Thaw the mixture in a water bath set at 35°C. Repeat this freeze-thaw process until performed five times in total. Assemble the extruder with a drain disc followed by two 0.4um membranes. Pass the lipid mixture through the extruder twice with the minimum of pressure from the

nitrogen line. The change the discs for two 0.2um discs and pass the lipid mixture through this time a total of ten times. The liposomes can then be stored in a fridge. The liposomes, though made, need to be purified to remove any excess enzyme that has not been encapsulated. This is performed by adding 100mg Trypsin attached to DITC glass beads to 1ml of liposomes and mixing for 11-12 hours at room temperature. The beads should be washed in demin prior to use to remove stabilizer. After the incubation period the beads are removed by centrifuging at 13,000rpm for 10 minutes. The liposomes are removed and stored at refrigeration temperatures.

In order to make the liposomes pH sensitive, the M-gala peptide has to be inserted into the lipid membrane. This is simply done by incubating the M-gala solution (0.1mg/ml) with the liposomes in equal volumes for 30 minutes.

3.1.2 Mode of Action.

pH changes caused by the growth of *Salmonella* cells producing acidity in the medium, cause the pH sensitive peptide to release the contents of the liposome. The release asparaginase enzyme reacts with the asparagine in the media to cause an increase in conductivity.

L-asparagine + H2O - L-asparate + NH3

3.1.3. Conductivity Experiments.

These experiments were carried out on a Don Whitley Scientific RABIT (Rapid Automated Bacterial Impedance Technique). The system uses individually assembled plastic cells with two electrodes in the bottom. The cells sit in a module maintained at 37°C attached to a computer from which the machine is run. Media (minimum 2mls) is placed in each cell and the system can be set to read the conductivity of each of the 32 cells every minute for up to 24 hours. Specifically formulated selective media which has a low conductivity is used, and as the target organism grows the bacteria cause the conductivity of the media to increase. This change in conductivity over time is plotted giving the appearance of a sigmoidal growth curve. When these pH sensitive liposomes are introduced to the system the liposomes are lysed as the bacteria grow and lower the pH, and hence the asparaginase/asparagine cause the conductivity of the solution to change faster than the action of the bacteria alone. In theory the use of the liposomes will give an accelerated detection time (approximately 2 hours) over the 8-24 hour detection for most *Salmonella* species using selective media alone.

3.1.4 Liposome controls.

Requirements.

Media - CRM or TMAO (see appendix for recipes). Asparaginase liposomes made as per protocol, 30mM Asparagine in demin (substrate), 0.00025mg/ml M-gala in demin (peptide), 10% triton in demin (detergent).

In order to check the quality of each batch of liposomes produced, a series of quality control checks were carried out each time. These included;

(1) Background assay.
2mls media, 100ul liposomes, 200ul substrate.
The conductivity graph of this mixture will indicate any gradual loss of enzyme from the liposomes.
(2) Lysis assay.
2mls media, 100ul liposomes, 200ul substrate, 10ul triton.
The triton (detergent) lyses the liposomes causing an immediate increase in conductivity, thus demonstrating that the liposomes contain sufficient enzyme to give a detectable signal.
(3) pH sensitive liposomes background.
2mls media, 100ul liposomes, 200ul substrate, 50ul peptide.
As the peptide inserts into the liposomes a slight increase in background should be seen. This control is used as a standard to compare experiments to. Acid production by growing cells should produce a significantly larger signal than this background assay.

3.1.5. *Salmonella* growth experiments.
For each of these experiments 2mls of media was introduced to a RABIT tube and allowed to stabilise for 30mins. After this time 200ul of substrate , 50ul of peptide and 100ul of liposomes were added to the tube along with a dilution of a bacterial culture. The modules were run at 37°C for between 8 and 24 hours taking readings every 1 minute. Over this time the Salmonella grew, produced acid, which cause lysis of the liposomes and hence an increase in conductivity. This increase in conductivity showed as a growth curve plotted by the RABIT software.

3.2 RESULTS

3.2.1 Salmonella growth curves.
As the liposome conductivity methodology was designed to accelerate the standard conductometric time to detection, the first experiments were aimed at determining this standard time to detection. In addition, devising media suitable for conductivity experiments within the measuring range of the equipment used, were required. Time to detection for the standard method was determined as the time taken for a sigmoidal growth curve to be seen. Four species of *Salmonella* were grown in TMAO at 37oC for 24 hours (see Fig 6). As expected, time to detection varied between species and was between about 6 hours for *S. enteritidis* and 20 hours for *S. abony*. Therefore, any improvement on these times that could be demonstrated by the liposome method, w

3.2.2 Liposome sensitivity tests.
The next set of experiments aimed at comparing the standard times to detection on the RABIT with the 'accelerated' liposome method. This was carried out using a dilution

series of an overnight *Salmonella* culture, as this also gave an indication of the numbers required for the accelerated method to be beneficial, i.e. the sensitivity of the method. If the accelerated methodology is working, the sigmoidal growth curve of the cultures plus the liposomes would be seen several hours before the growth curve for the standard experiment. This set of experiments were carried out several times, one set of results only are shown (see figs 7, 8 and 9).

The results show that for the 10^{-1} dilution of *S. enteritidis* (approximately 10^{8} cfu/ml), the logarithmic growth phase was detected at about 5 hours for the liposome experiment and 7 hours for the control. The 10^{-2} dilution (10^{7} cfu/ml) again showed an approximate 2 hour acceleration on the standard method. However, the 10^{-3} dilution had not even started its logarithmic growth phase at the end of the 8 hour experiment, indicating that the possibility of a greatly accelerated test giving results in a single day for low numbers of target organisms, was unlikely without considerable further development of the method.

3.2.3. Control Experiments.
With each set of experiments, control tubes were run as outlined in 3.1.4. A typical set of results follows (see fig 10).

This graph shows the media control (line 1) with a steadily increasing reading though at a low level. (The variations in this largely straight line were caused by an error in the Rabit itself that engineers were unable to alleviate). Line 2 shows the liposome control and gives an indication of the background of the liposomes. Line 3 is the triton lysis of the liposomes and this shows a steep rise over the first two hours followed by a levelling off caused by the fact that all the liposomes are lysed and there is substrate limitation.

These controls were carried out each time. At the beginning of the work at LGC these controls were regularly showing a high background signal for the liposome control caused either by the leakage of enzyme from the liposomes or from excess enzyme bound to the outside of the liposome. This resulted in considerable efforts being invested by LGC and Manchester to try to resolve the problem. Eventually the problem was traced to detergent residues in the rabit tubes causing lysis. Overnight soaks of the tubes in demin water was sufficient to resolve this problem, though it did highlight the sensitivity of this method to detergent residues. Also during this stage of the work a number of methods were tested in order to reduce the background signal of the liposomes (see section 3.2.4).

3.2.4. Method development.
The conductivity evaluation work at LGC lasted a total of 9 months, though a considerable amount of this time was spent in technology transfer of the methodology and method development (this work has been previously reported in the fourth interim report to MAFF). During the 9 months of work a number of developments to the method were made, and these are summarised here;

(1) Optimisation of the growth media used for the conductometric tests to ensure that the conductivity fell within the detectable limits of machines used at CAMR and LGC.
(2) Assessment of the affects of filter sterilising reagents on assay performance.
(3) Assessment of the amount of peptide used for optimal signal/background ratio.
(4) Optimisation of the amount of liposomes used per experiment for distinct signal to be seen.
(5) Purification methods for liposomes using sepharose CL6B and trypsin beads were tested in order to reduce the background signal caused by surface bound enzyme.
(6) Changes to the liposome production method.
(7) Storage conditions and expiry times of reagents.

The liposome sensitivity test results reported here were achieved with the method encorporating each of these optimised issues. Even so the method was still only demonstrating acceleration of approximately 2 hours on the standard methodology. Following a review meeting with the MAFF project monitoring officer in January 1996, a decision was taken to move from the conductometric method in broth culture to the alternative pH methodology on agar plates. The results of this work are discussed in the next section.

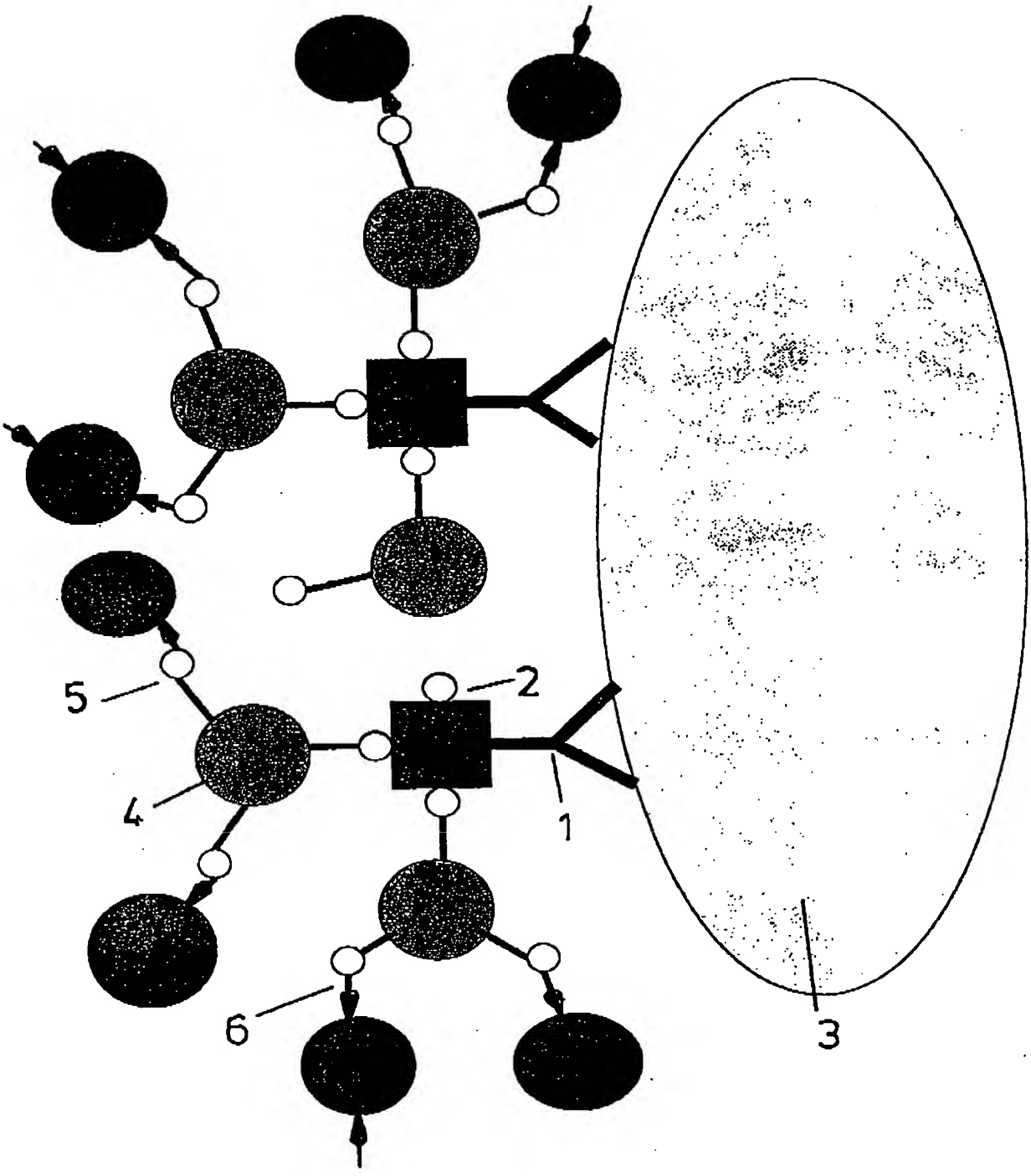


FIG.1

Figure 2

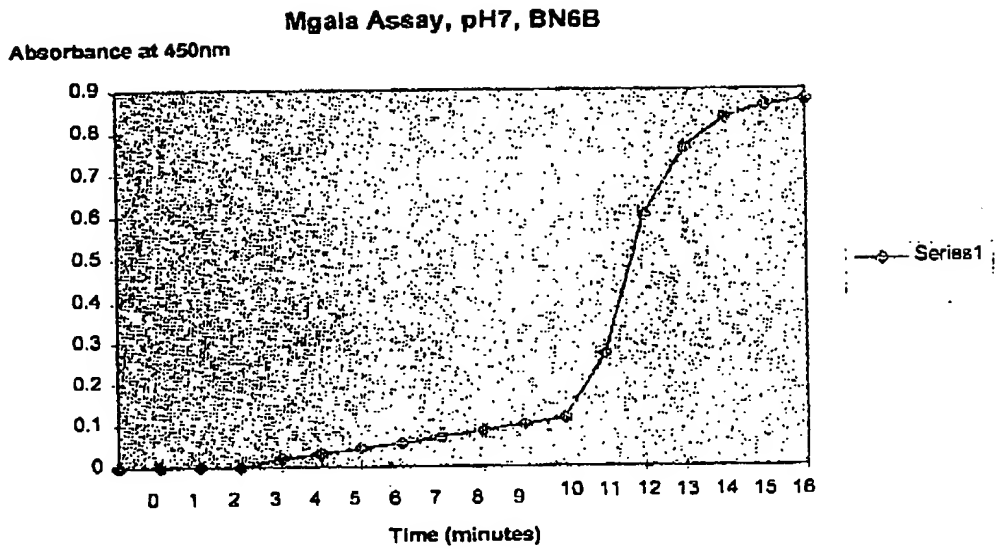


Figure 3

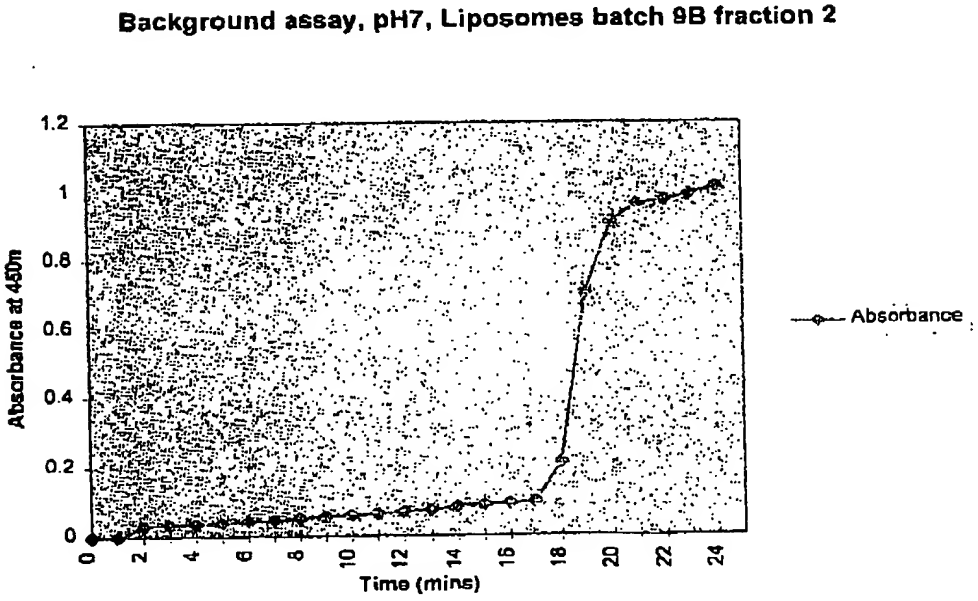


Figure 4

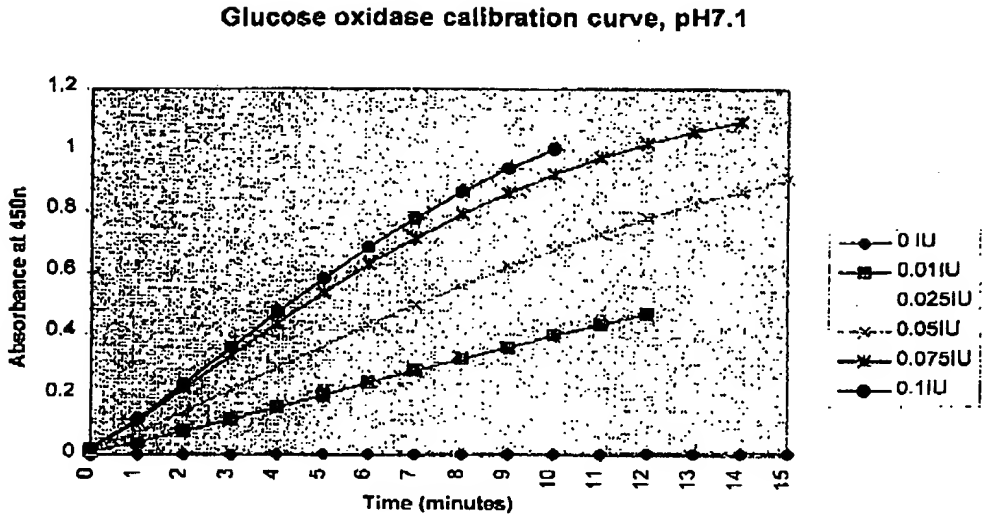
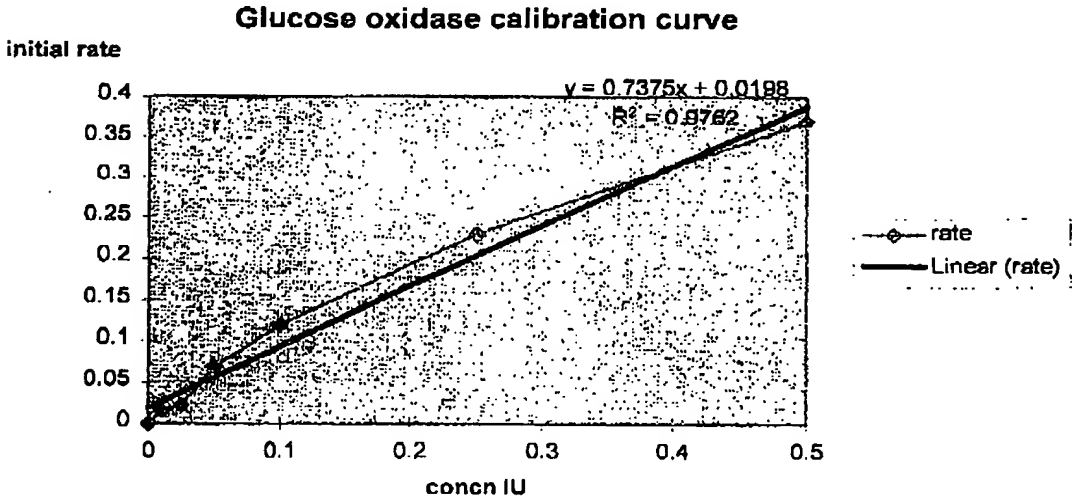


Figure 5



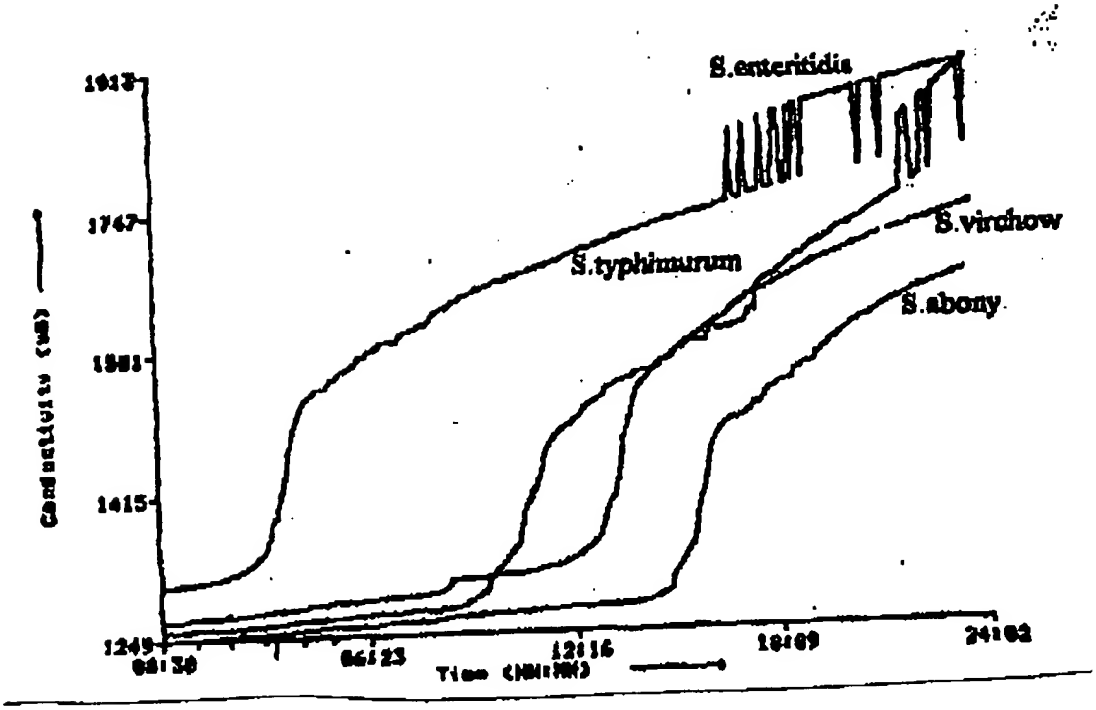

S.enteritidis
S.virchow
S.typhimurum
S.abony
1747
1581
1415
1249
Time (HH:MM)

Fig 6.

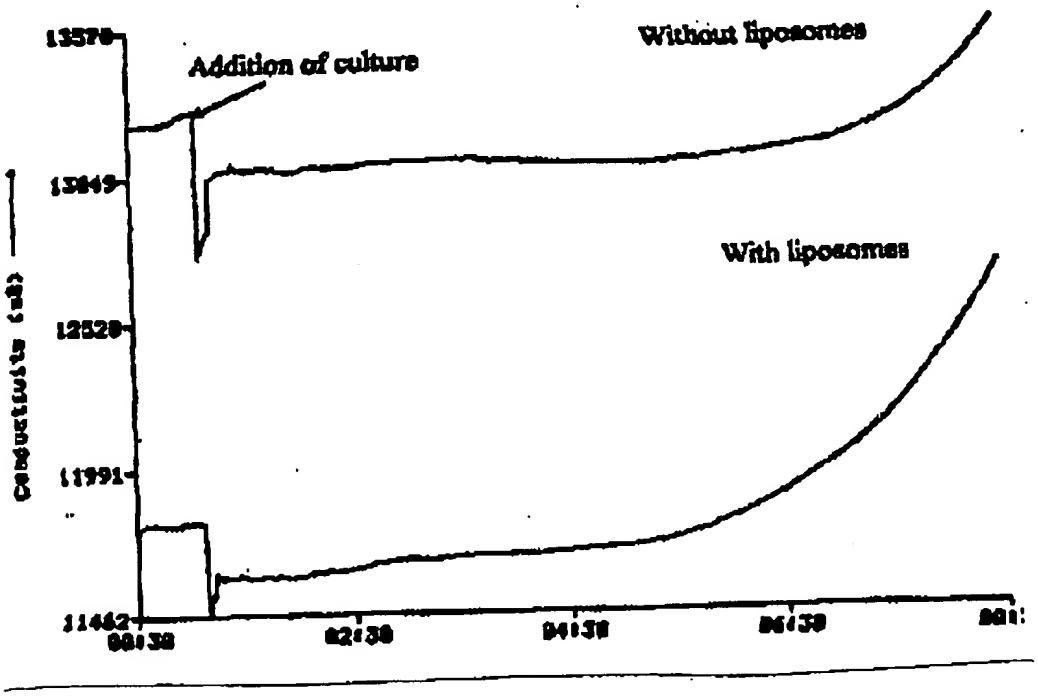

Addition of culture
Without liposomes
With liposomes
13849
12520
11991
11462

Fig. 7

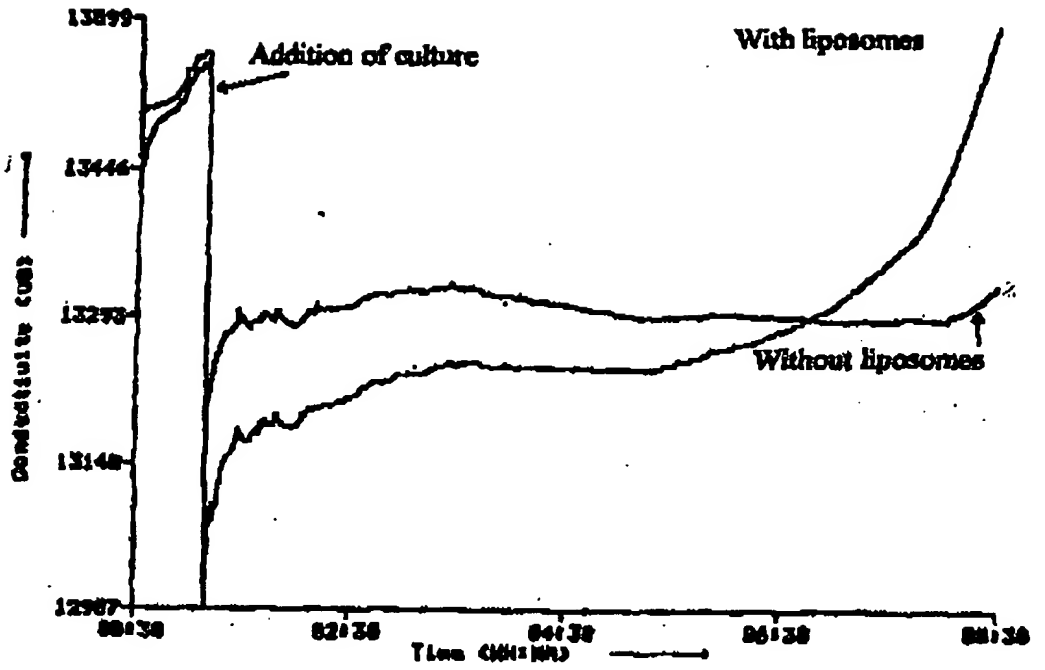

Addition of culture
With liposomes
Without liposomes

Fig 8.

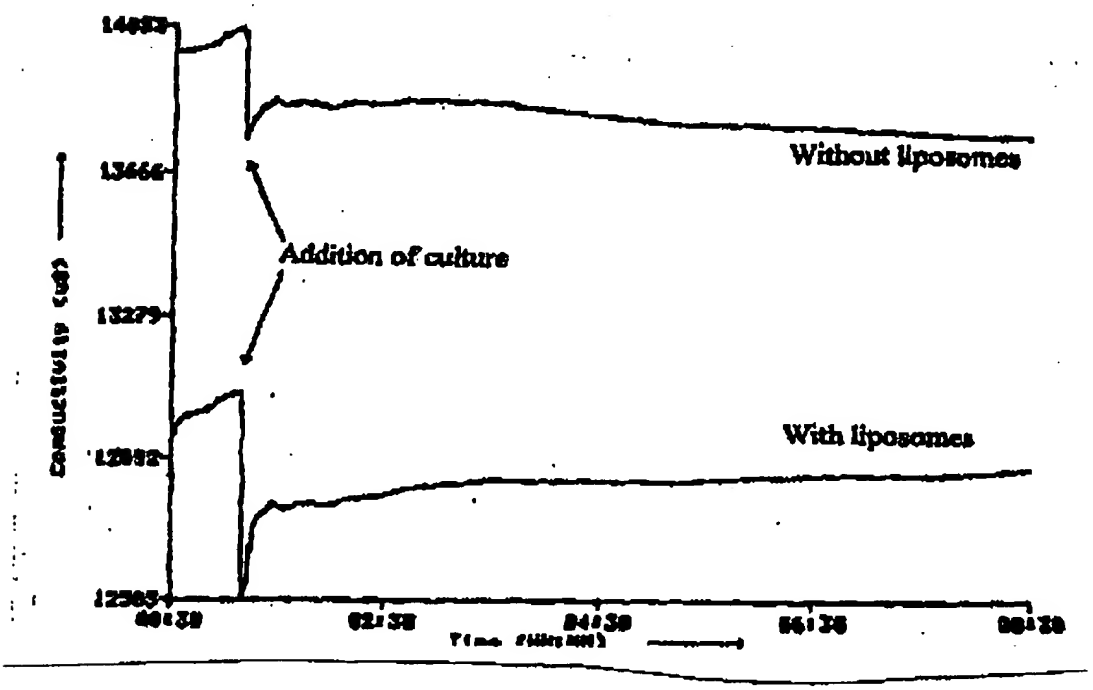

Without liposomes
Addition of culture
With liposomes

Fig 9.

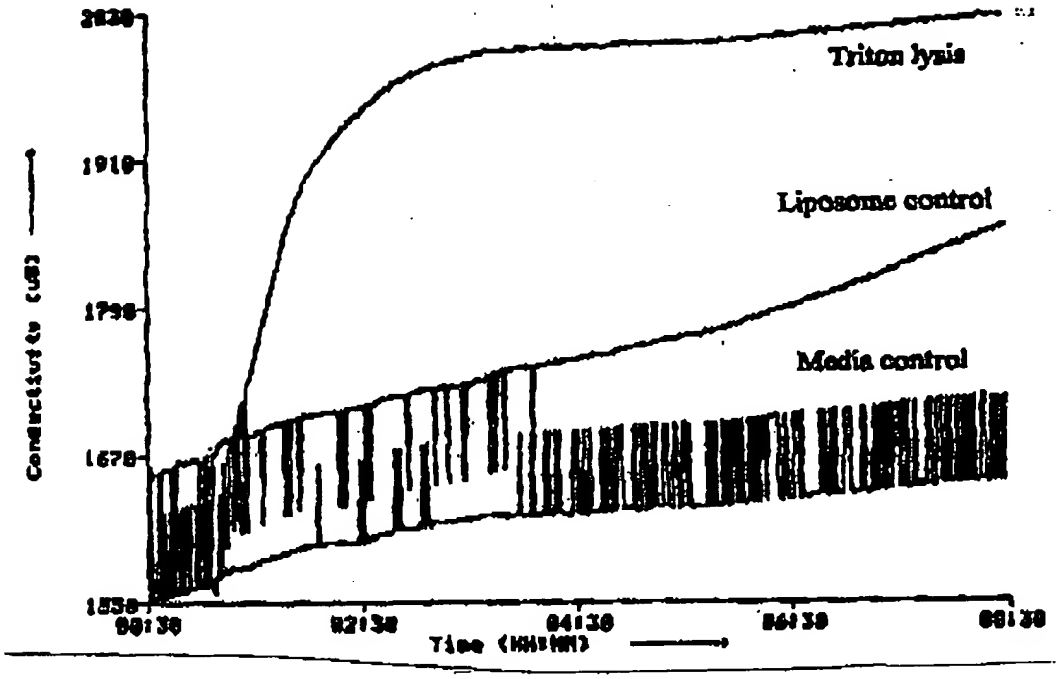

Triton lysis
Liposome control
Media control
1918
1798
1678
1558
Conductivity (uS)
00:30
02:30
04:30
06:30
08:30
Time (HH:MM)

Fig. 10.

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